VOLUME 9 May 1960 NUMBER 3

THE AMERICAN JOURNAL OF Tropical Medicine and Hygiene

Combining The American Journal of Tropical Medicine and The Journal of the National Malaria Society

OFFICIAL ORGAN OF
THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

PUBLISHED BIMONTHLY BY
THE WILLIAMS & WILKINS COMPANY
BALTIMORE 2, MARYLAND, U.S.A.

© Copyright 1960, By The American Society of Tropical Medicine and Hygiene
Made in U.S.A.

THE AMERICAN JOURNAL OF

Tropical Medicine and Hygiene

Editor-in-Chief
PAUL C. BEAVER
1430 Tulane Avenue
New Orleans 12, Louisiana

Associate Editor
MARTIN FROBISHER
Box 91
Harwich, Massachusetts

Editor, Tropical Medicine and Hygiene News
WILLIAM B. DEWITT, National Institutes of Health
Bethesda 14, Maryland

Editorial Board

WILLIAM McD. HAMMON
JOHN A. LOGAN
HARRY MOST
LLOYD E. ROZEBOOM
ALBERT B. SABIN
LEON H. SCHMIDT
THOMAS H. WELLER
MARTIN D. YOUNG

INFORMATION FOR SUBSCRIBERS

THE AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE is issued bimonthly, appearing in January, March, May, July, September, and November. One volume is issued per year.

Correspondence concerning business matters, subscriptions and orders for reprints, should be addressed to The Williams & Wilkins Company, 428 East Preston Street, Baltimore 2, Maryland, U. S. A.

Manuscripts should be sent to Dr. Paul C. Beaver, 1430 Tulane Avenue, New Orleans 12, Louisiana. *Books for Review* should be sent to Dr. Martin Frobisher, Box 91, Harwich, Massachusetts.

New subscriptions and renewals are entered to begin with the first issue of the current volume. Should any issue of the current volume be out of print at the time the subscription order is received, the pro rata value of such numbers will be refunded to the subscriber.

Subscriptions should be renewed promptly—To avoid a break in your series, subscriptions should be renewed promptly. The publishers cannot guarantee to supply back issues on belated renewals.

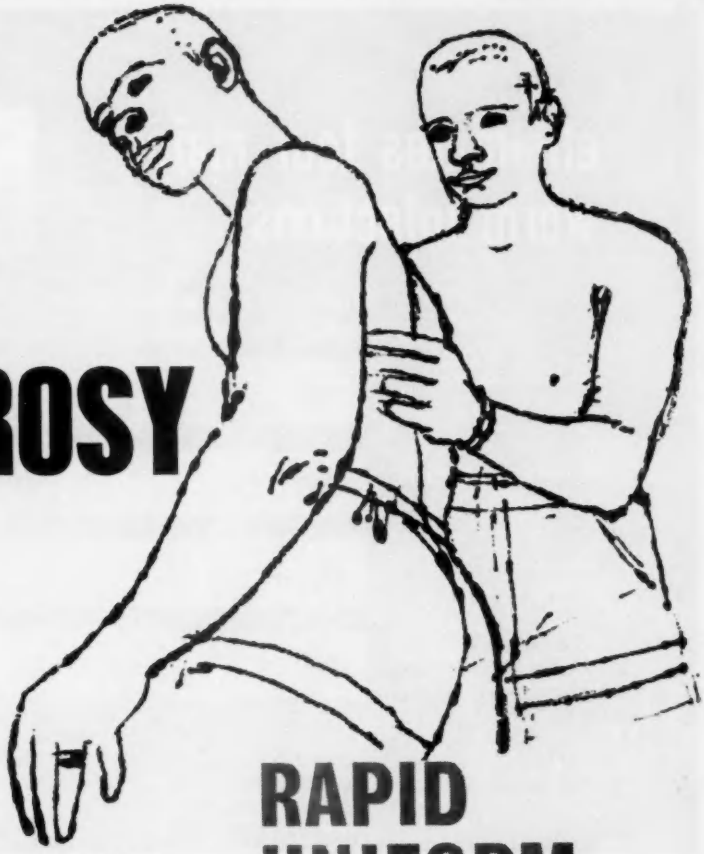
Change of address: Publisher must be notified 60 days in advance. Journals undeliverable because of incorrect address will be destroyed. Duplicates can be obtained (if available) from the publisher at the regular price of single issues.

OFFICERS OF THE
American Society of Tropical Medicine and Hygiene

President	*Councilors:*	*Term Expires*
WILLARD H. WRIGHT	JOHN M. HENDERSON	1960
President-Elect	J. ALLEN SCOTT	1960
WILLIAM W. FRYE	PAUL F. RUSSELL	1961
Vice-President	JOSEPH E. SMADEL	1961
G. ROBERT COATNEY	MARSHALL HERTIG	1962
Secretary-Treasurer	JOHN E. LARSH, JR.	1962
ROLLA B. HILL	ARNOLDO GABALDON	1963
3575 St. Gaudens Road	HARRY MOST	1963
Miami 33, Florida		

Individuals interested in any phase of tropical medicine and hygiene, including all arthropod-borne diseases, medical care and human welfare are eligible for membership. Dues are $10.00 a year and include subscription to the American Journal of Tropical Medicine and Hygiene. An application blank is bound into this issue of the Journal for the use of interested individuals. Before being submitted to the Secretary, each application must be signed by two active members of the Society and a check for $10.00 (U.S. banks preferred) must accompany the application.

Published bimonthly by The Williams & Wilkins Co., 428 East Preston Street, Baltimore 2, Md., U.S.A.
Subscription price $10.00, single issue $2.00
Second-class privilege authorized at Baltimore, Md.



IN LEPROSY

RAPID UNIFORM PROGRESS WITH ETISUL

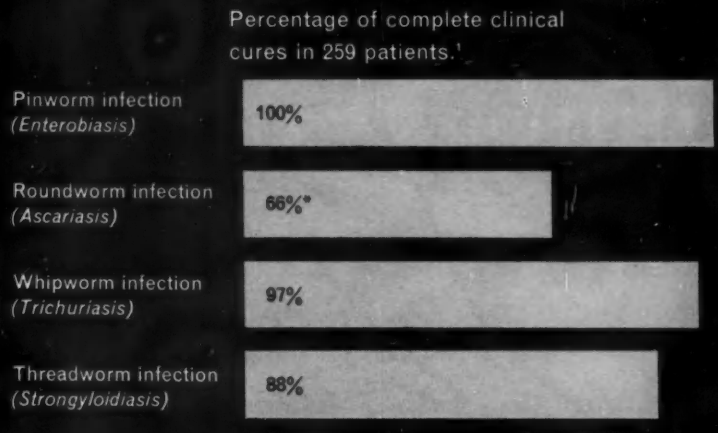
Trade Mark

Percutaneous treatment—psychologically sound free from side effects

ICI

IMPERIAL CHEMICAL INDUSTRIES LIMITED PHARMACEUTICALS DIVISION Wilmslow Cheshire England

eliminates four major worm infections



*Although one-third of the patients with roundworm infections were not completely cured, Delvex produced a 97 percent reduction in fecal egg counts.

DELVEX®, the first wide-spectrum anthelmintic

Clinical studies show:

- Delvex is effective orally, usually within five days, against four of the five most common worm infections:
  Pinworm Whipworm
  Roundworm Strongyloidiasis
- It is partially effective, and sometimes curative, in hookworm infection.
- It is fully effective in both single and multiple infections and in both heavy and light infections.
- In optimal dosage, it eliminates pinworm infection in 100 percent of patients.
- It is the first effective and practicable agent for the oral treatment of strongyloidiasis and whipworm infection.
- No adjunctive measures are needed with Delvex therapy.

Further information and clinical reports may be obtained from your Lilly representative or by writing to our medical division.

1. Swartzwelder, J. C., et al.: J.A.M.A., 165:2063, 1957.

Delvex® (dithiazanine iodide, Lilly)

ELI LILLY AND COMPANY • INDIANAPOLIS 6, INDIANA, U.S.A.

060614

THE AMERICAN JOURNAL OF

TROPICAL MEDICINE AND HYGIENE

Vol. 9 *May 1960* *No. 3*

CONTENTS

Rice-field leptospirosis in Turkey. A serologic survey. **Walter E. Brewer, A. D. Alexander, Fahri Hakioglu and L. B. Evans** ... 229

Distribution of eggs of different geographic strains of *Schistosoma japonicum* in the viscera of infected hamsters and mice. **H. F. Hsü and S. Y. Li Hsü** ... 240

Studies on echinococcosis: Serology of crude and fractionated antigens prepared from *Echinococcus granulosus* and *Echinococcus multilocularis*. **Irving G. Kagan, Lois Norman and Dorothy S. Allain** ... 248

Prevalence of intestinal protozoa in adults at four military bases in California. **R. L. Brown and M. J. Garber** ... 262

Alteration of the environment of *Anopheles* larvae by nutria in the Naaman Swamps, Acre District, Israel. **S. Ehrlich and D. Spielberg** ... 265

Spontaneous acute toxoplasmosis in a marmoset monkey. **Kurt Benirschke and Ralph Richart** ... 269

A study of ascariasis eradication by repeated mass treatment. **Francisco Biagi F. and Oscar Rodriguez** ... 274

The effect of metabolites on the antitoxoplasmic action of pyrimethamine and sulfadiazine. **Don E. Eyles and Nell Coleman** ... 277

Active immunization of man against the venom of the Australian tiger snake (*Notechis scutatus*). **Saul Wiener** ... 284

The effect of anti-erythrocytic antibodies upon *Plasmodium berghei* infections in white mice. **Thomas M. Schwink** ... 293

A note on psychoses and Loeffler's syndrome complicating creeping eruption. **Morris W. Dexter, Helen L. T. Dexter and Alfred H. Lawton** ... 297

Serologic diagnosis of *Schistosoma mansoni* infections. I. Development of a cercarial antigen slide flocculation test. **Robert I. Anderson** ... 299

Poliovirus infection in Guatemala. **Margaret I. Sellers, A. F. Rasmussen, Jr. and Nevin S. Scrimshaw** ... 304

The effect of folic acid on the steatorrhea of tropical sprue and other tests for intestinal absorption. **R. Rodriguez-Molina, Marta Cancio and Conrado F. Asenjo** ... 308

Infectivity to mosquitoes of *Plasmodium vivax* and *Plasmodium falciparum* under various conditions. **Geoffrey M. Jeffery** ... 315

The physiology of hibernation and its role in the geographical distribution of populations of the *Culex pipiens* complex. **Aseffa Tekle** ... 321

The effect of temperature and illumination on mating of *Culex pipiens pipiens* L. and *C. p. fatigans* Wied. **Anne C. M. Parker and Lloyd E. Rozeboom** ... 331

Observation on etiology of diarrheal diseases in Arizona. **Melvin H. Goodwin, Jr., Don C. Mackel, Robert S. Ganelin, Robert E. Weaver and Fred J. Payne** ... 336

Studies on paralytic poliomyelitis in Cairo, U.A.R., 1957–1958. **Thomas G. Akers, Nemat Hashem and M. Sabet Mahdy** ... 343

Books Received ... 350

Book reviews: *The Ecology of Human Disease*, by May; *Parasitology (Protozoology and Helminthology) in Relation to Clinical Medicine*, by Chatterjee; *A Textbook of Medicine*, Cecil and Loeb, ed'rs; *Assistance Technique en Médecine Africaine*, by Devignat; *Blindness in West Africa*, by Rodger; *Report of the First Institute on Clinical Teaching: 6th AAMC Teaching Institute*, Gee, Richmond and Nourse, ed'rs; *Water Supply for Rural Areas and Small Communities*, by Wagner and Lanoix; *Le Paludisme dans la Zone Pilote Antipaludique de Bobo Dioulasso (Haute-Volta, A.O.F.)*, by Choumara, Hamon, et al. ... 350

Coordinates both clinical and preventive medicine . .

Morris: USES OF EPIDEMIOLOGY

This book is addressed to students of both clinical and preventive medicine. It attempts to indicate how epidemiology can serve as a means of bringing these two fields together. However, only passing reference is made to the infections, and the author suggests that *Some Uses of Epidemiology in the Study of Noncommunicable Diseases* might be a more accurate title.

Contents: INTRODUCTION; I. HISTORICAL STUDY—Future Population Trends; II. COMMUNITY DIAGNOSIS—Note on Procedures (1), Changing People in a Changing Society; III. WORKING OF HEALTH SERVICE—Distribution of Services, Quality of Medical Care, Needs, Changing Needs; IV. INDIVIDUAL CHANCES—Epidemiology of Personal Habits; V. COMPLETING THE CLINICAL PICTURE—Completing the Picture in Breadth, Completing the Picture in Depth, "Medicine Outside the Hospital", Early Detection of Disease, Completing the Picture in Time, In space, The Appropriate "Universe" in Physiological Studies; VI. IDENTIFICATION OF SYNDROMES—Association of Diseases; VII. IN SEARCH OF CAUSES—Modern Epidemic, Multiple Causes, Note on Procedures (2), Prospective Studies, Unhealthiness of Towns, Dangerous Trades, Respiratory Infection in Infants, Cancer in the Reports of the General Register Office, Geographical Pathology, Ecology of Mental Disorders, Experimental Epidemiology; RECAPITULATION—GENERAL; APPENDIX—COMPLEMENTARY NATURE OF CLINICAL, LABORATORY AND POPULATION STUDIES—Production of Congenital Malformation by Rubella, Causes of Cancers, Solving a Nutritional Problem, Outbreaks of Food Poisoning Associated with Canteen Meals, Unexplained High Stillbirth Rate, Familial Factors in Disease, Natural History of Disease, Two Types of Diabetes, Discovery of a New Disease, Reality of an Old One, Population Genetics, Organism-in-Environment, Balance, Equilibrium, Exercise of Mental Functions, Multiple Causality, Provision of Hearing Aids, Causes and Prevention of Industrial Injuries; REFERENCES—GENERAL—References—Special; INDEX OF SUBJECTS.

By J. N. MORRIS, *M.A., M.R.C.P., D.P.H., Director, Social Medicine Research Unit of the Medical Research Council, London Hospital*

1957 • 143 pp. 28 figs. • **$4.00**

Publishers of Medical and Scientific Books and Periodicals

SANS TACHE

WILLIAMS & WILKINS COMPANY

BALTIMORE 2, MD.

SHOP BY MAIL

THE WILLIAMS AND WILKINS COMPANY
Baltimore 2, Md.

Please send the following on approval:

_____

_____

Name_____ (Please print)

Address_____

City_____ Zone_____ State_____

☐ Payment enclosed. ☐ Bill me.

Shopping by mail is an easy, time-saving way to select books for your personal library.

FROM PARKE-DAVIS
A NEW ORAL ANTIBIOTIC

EFFECTIVE ANTIBACTERIAL AND ANTIAMEBIC ACTIONS

● KAPSEALS®

Humatin®

(PAROMOMYCIN, PARKE-DAVIS)

- **IN INFECTIOUS DIARRHEAS**
- **IN INTESTINAL AMEBIASIS**
- **IN PREOPERATIVE SUPPRESSION OF INTESTINAL FLORA**
- **IN MANAGEMENT OF HEPATIC COMA**

Humatin

KAPSEALS

(PAROMOMYCIN, PARKE-DAVIS)

EFFECTIVE—WELL TOLERATED

A newly developed antibiotic for oral administration, HUMATIN exhibits broad antibacterial and effective antiamebic activity—*in vitro*,[1-3] against experimental infections in animals,[1-3] and in clinical studies.[1,4-9] Its therapeutic effectiveness and the desirable characteristic of being virtually unabsorbed from the intestinal tract[4] make HUMATIN an antibiotic of choice in a variety of enteric infections.
Since HUMATIN by the oral route is virtually unabsorbed from the intestinal tract even with exceptionally high doses, systemic toxicity has not been a problem in clinical use.[1,4-9]

IN INFECTIOUS DIARRHEAS

Because it is effective against certain gram-negative pathogens, HUMATIN has proved particularly useful in enteric infections, most of which are caused by bacilli of the gram-negative group. In 85 per cent of 221 adults and children with severe dysenteries, remission of symptoms was rapid with HUMATIN; stools cleared quickly of pathogens, and remained negative for reasonable periods following cessation of therapy. *Escherichia coli* proved most susceptible to HUMATIN; 95 per cent of such infections responded to treatment within a few days. *Shigella* responded in more than 88 per cent of cases, and *Salmonella* in over 80 per cent. Side effects were few and of a minor nature; they were reported in less than 5 per cent of patients.[5]

IN INTESTINAL AMEBIASIS

Amebiasis, endemic in the tropics, and probably present in more than 10 per cent of the population of the United States,[10] has responded to HUMATIN "...within three to eight days...."[1] The drug is unusually effective in clearing all phases of intestinal amebiasis—acute, subacute, and chronic.[1,5]
Since HUMATIN is not appreciably absorbed from the gastrointestinal tract, it is not effective against extraintestinal forms of amebiasis.
In 311 patients, striking symptomatic relief was obtained in virtually all as early as the second or third day of treatment. Clinical cures were achieved in 95 per cent of those receiving adequate dosage.[5]
In another study, all 20 patients obtained prompt relief; "...diarrhea stopped in three to eight days; local tenderness overlying a segment of the colon was relieved in two to seven days; intestinal ulcerations healed in 4 to 10 days, and the stools were negative within three to seven days."[1]
In two other groups of 31[8] and 79[9] patients, respectively, relief of symptoms was prompt, and cysts were rapidly eradicated from the stools.

IN SUPPRESSION OF INTESTINAL FLORA PRIOR TO BOWEL SURGERY

In preliminary studies, HUMATIN showed promise of being an effective agent for lowering the bacterial count of the gastrointestinal tract prior to bowel surgery. Therapy was followed by a prompt decline

in total aerobes, coliform bacilli, and total anaerobes. Growth of staphylococci remained relatively constant; in no instance did staphylococcal superinfection present a problem.[5]

IN MANAGEMENT OF HEPATIC COMA

Although the exact mechanism underlying the onset of hepatic coma has not yet been fully established,[11] there can be no doubt of the value of certain nonabsorbable antibiotics in the adjunctive management of this condition. In animals, antibiotics have been shown to reduce the amount of ammonia produced and absorbed from the colon;[12] this effect is probably a factor in the "...dramatic response and complete recovery..."[4] so frequently seen following the administration of HUMATIN in adequate dosage.[4-7]

ADMINISTRATION AND DOSAGE (expressed in terms of base)

Condition	*Dosage (daily, in divided doses)*	*Duration of Therapy*
Infectious Diarrheas (bacillary and nonspecific)	*Adults*—35 to 50 mg/Kg; up to 100 mg/Kg in severe infections.	Up to 7 days
	Children—50 mg/Kg; up to 100 mg/Kg in severe infections.	Up to 7 days
Intestinal Amebiasis (acute, subacute, chronic)	*Adults*—0.75 to 1.5 Gm.; larger doses when required.	5 days
	Children—22 mg/Kg; larger doses when required.	5 days
Preoperative Suppression of Intestinal Flora	*Adults*—2 Gm.	4 days
Hepatic Coma	*Adults*—up to 6 Gm., depending on degree of hepatic insufficiency and response of patient.	See literature

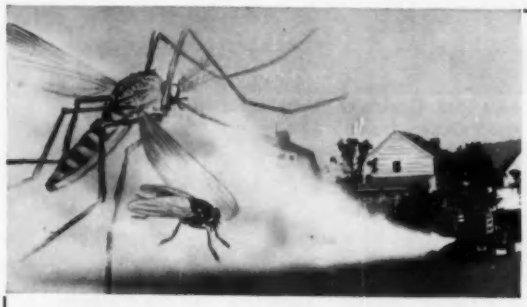
SIDE EFFECTS: Since HUMATIN is relatively nonabsorbable in the gastrointestinal tract, side effects are rarely encountered. However, when doses in excess of 2 Gm. per day are given for more than three days, loose stools may develop; on doses of from 4 to 6 Gm. daily, moderately severe diarrhea has been reported in some patients. No other indications of toxicity have been observed.

SUPPLIED: HUMATIN (paromomycin, Parke-Davis) is available as the sulfate in Kapseals,® each containing 250 mg. of base; bottles of 16. Literature supplying details of dosage and administration available on request.

References: (1) Shafei, A. Z.: *Antibiotic Med. & Clin. Therapy* 6:275, 1959. (2) Coffey, G. L., *et al.: Antibiotics & Chemother.* 9:730, 1959. (3) Thompson, P. E., *et al.: Antibiotics & Chemother.* 9:618, 1959. (4) Fast, B. B., *et al.: Arch. Int. Med.* 101:467, 1958. (5) Personal Communications to Department of Clinical Investigation, Parke, Davis & Company, 1959. (6) Mackie, J. E., *et al.: New England J. Med.* 259:1151, 1958. (7) Stormont, J. M., *et al.: New England J. Med.* 259:1145, 1958. (8) Elias, F. L., & Oliver-Gonzalez, J.: *Antibiotic Med. & Clin. Therapy* 6:584, 1959. (9) Carter, C. H.: *Antibiotic Med. & Clin. Therapy* 6:586, 1959. (10) Faust, E. C., & Russell, P. F.: Craig and Faust's Clinical Parasitology, ed. 6, Philadelphia, Lea & Febiger, 1957, p. 198. (11) Editorial: *New England J. Med.* 259:1181, 1958. (12) Silen, W., *et al.: Proc. Soc. Exper. Biol. & Med.* 88:138, 1955.

PARKE, DAVIS & COMPANY
DETROIT 32, MICHIGAN

PARKE-DAVIS



MOSQUITOES, FLIES AND OTHER INSECT PESTS

DESTROYED ON CONTACT

TIFA

NOW SUITABLE FOR OPERATION ON PROPANE OR GASOLINE

Best by test for over 15 years . . . on the dump, over pools, in suburbs, play areas, stockyards, everywhere that insects swarm TIFA, lowest of all in operating and maintenance costs, rapidly covers large areas with true clean fog. Maximum lethal efficiency with newest or older type insecticides. One-man operation. Starter-generator optional.

TODD INSECTICIDAL FOG APPLICATOR

OVER 15 YEARS successful world-wide performance in Public Health programs of more than 5,000 communities.

Carrying Underwriters' Listing Seal No. MH 4162.

Write for illustrated folder and official comments.

TODD SHIPYARDS CORPORATION

PRODUCTS DIVISION

SALES AND SERVICE DEPARTMENTS Columbia and Halleck Streets, Brooklyn 31, N. Y.

PLANT, SALES AND SERVICE Houston, Texas

Sales and Service Agencies throughout the Western Hemisphere

THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE

APPLICATION FOR MEMBERSHIP

To the Membership Committee:

The following individual interested in tropical medicine and hygiene is proposed for active membership

(TYPE OR PRINT)

Name ..... (*Last*) ..... (*First*) ..... (*Middle*)

Mailing Address: Street ..... City .....

State ..... Country .....

Date of Birth ..... Place .....

Present Position .....

Special Field of Interest .....

Education: Institution (Graduate and Postgraduate) Degree Date

.....

.....

.....

Attach to the application a list of professional positions held, membership in scientific societies, and a list of major publications.

* Proposed by:

(1) .....

(2) .....

Signature of Applicant

* Two active members must sign this application.

Date .....

Return this application to Dr. Rolla B. Hill, Secretary-Treasurer, 3575 St. Gaudens Road, Miami 33, Florida

(Check for $10.00 (U. S. banks requested) must accompany this application.)

NATIONAL® BIOLOGICAL STAINS for FLUORESCENCE MICROSCOPY

Already showing considerable promise in expediting precise diagnoses, the use of fluorescence microscopy should grow as a wider spectrum of fluorescent biological stains becomes available and new techniques for their effective use are developed.

Recognizing the growing importance of these diagnostic aids, National can supply through nearby medical supplyhouse stocks:

#408 National ACRIDINE ORANGE

as well as these fluorescent National Biological Stains

#545 FLUORESCEIN — FREE ACID **#544 FLUORESCEIN SODIUM U.S.P.**

#661 RHODAMINE B **#702 RHODAMINE B Base** **#748 ACID RHODAMIN B**

And to assist you in specifying stains, indicators and reagents—

We will be pleased to send you a copy of our current 60-page catalog giving code numbers, packaging and prices on our comprehensive line of Laboratory Reagents; Biological Stains and Reagents; Commission Certified Biological Stains; Staining Solutions and Reagents in Solutions, as well as National Indicators and Oxidation-Reduction Indicators. This book also contains a Hydrogen-Ion Indicator Chart and other useful information.

NATIONAL ANILINE DIVISION
40 RECTOR STREET, NEW YORK 6, N. Y.
Atlanta Boston Charlotte Chicago Greensboro Los Angeles
Philadelphia Portland, Ore. Providence San Francisco
In Canada: ALLIED CHEMICAL CANADA, LTD., 100 North Queen St., Toronto 18
Distributors throughout the world. For information:
ALLIED CHEMICAL INTERNATIONAL • 40 Rector St., New York 6, N. Y.

Allied Chemical

A sociological and medical study of bejel. . .

Hudson: NON-VENEREAL SYPHILIS

A study of the disease bejel in sociological and medical terms, by a world authority in treponemal infections. Advocates the recognition of syphilis and yaws as components of world-wide treponematosis, with bejel as an example of endemic syphilis which stands between them and draws them together. Of interest not only to the specialist in syphilis and tropical medicine, but also to the general physician, the public health officer, the lay health worker, the biologist, the sociologist, the anthropologist, and the student of geography of diseases.

Contents include: Treponematosis, the "Twisted Thread" Infection; Historical Perspective of Treponematosis; Bejel and Franji; Three Social Groups; Sex and Syphilis in the Three Social Groups; Incidence of Treponematosis; Venereal Lesions and Infections; Early Stage; Clinical Picture of Early Stage; Transmission of Bejel; Latent Period; Late Stage; Clinical Picture of Late Stage; Special Features of Late Stage; Effect of Bejel on Next Generation; Serology of Bejel (By Maunu Tuomioja, M.D., Helsinki); Microscopic Pathology of Bejel (By Richard E. Olsen, M.D., Pontiac, Michigan); Experimental Pathology of Bejel (Excerpts from T. B. Turner, M.D. and D. H. Hollander, M.D., Baltimore); Treatment of Bejel; Significance of Bejel; A New Social Hygiene.

By ELLIS HERNDON HUDSON, *M.D., D.T.M. & H. (Lond.), F.A.C.P., Emeritus Professor and Director of Health, Ohio University*

212 pp., 91 figs. (1957) • Price: $7.00

Publishers of Medical and Scientific Books and Periodicals

SANS TACHE

WILLIAMS & WILKINS COMPANY

BALTIMORE 2, MD.

SHOP BY MAIL

THE WILLIAMS AND WILKINS COMPANY
Baltimore 2, Md.

Please send the following on approval:

_____

_____

Name_____
(Please print)

Address_____

City_____ Zone____ State_____

☐ Payment enclosed. ☐ Bill me.

Shopping by mail is an easy, time-saving way to select books for your personal library.

Hastening the decline of malaria

'DARAPRIM'® brand
Pyrimethamine

"... the most efficient suppressive agent known, ..."

"... of extraordinary effectiveness against erythrocytic parasites of all the malarias."

"... probably has some action against the primary exo-erythrocytic forms of all the malarias, ..."

"... is active against the late tissue forms of *Plasmodium vivax* ..."

"... exhibits a phenomenal effect on gametocytes. ... the parasites are not able to develop beyond the early oöcyst stage, thereby blocking the chain of transmission."

Coatney, G. R. : Industry and Tropical Health, Volume II, 1955.

For the treatment of an acute attack 'Daraprim' should be supplemented with a fast-acting schizontocide.

safe • *economical* • *tasteless*

Full information will be sent on request.

Tablet of 25 mg.
Supplied in boxes of 30 and bottles of 1,000.

BURROUGHS WELLCOME & CO. (U.S.A.) INC., Tuckahoe, New York

THE MIRACLES OF MODERN INSECT CONTROL

From fields in which it was impossible to grow corn ten years ago, farmers now get 100 bushels per acre without rotation. Cotton fields that weren't worth picking now produce two bales per acre. Homes that would have rotted away from termite damage stand as sound as the day they were built. Lawns and gardens flourish, free of insect pests. These are some of the "miracles" of modern insecticides, and of the dedicated scientists who have developed safe and effective ways to use them. New pesticide chemicals have helped man increase his standard of living, his security, and his peace of mind. Those produced by Velsicol Chemical Corporation have been exceptionally useful. Velsicol is proud of them, and both grateful and indebted to the entomologists and other technicians who have made these insecticides so universally beneficial.

CHLORDANE
America's leading lawn and garden insecticide

HEPTACHLOR
America's leading soil insecticide

ENDRIN
A new weapon against hard-to-kill insects

VELSICOL

VELSICOL CHEMICAL CORPORATION
330 East Grand Avenue, Chicago 11, Illinois
International Representative: Velsicol International Corporation, C. A., P. O. Box 1687—Nassau, Bahamas, B.W.I.

VC 2787-968

RICE-FIELD LEPTOSPIROSIS IN TURKEY. A SEROLOGIC SURVEY

WALTER E. BREWER,* A. D. ALEXANDER,† FAHRI HAKIOGLU‡ AND L. B. EVANS†

Fourth Epidemiological Flight, U. S. Air Force, Izmir Turkey and Division of Veterinary Medicine, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

The cultivation of rice frequently provides optimum conditions for the spread of leptospirosis in man and animals. The crops attract large numbers of rodents—the natural hosts and carriers of leptospirosis—who excrete the infective agents onto plants and into the stagnant inundated fields. The organisms invade mammalian hosts through abrasions or cuts in the skin or through the ocular or nasopharyngeal mucous membranes. Infections frequently are acquired by contact with water or damp soil contaminated with animal urine. However, infections through direct contact with infective urine are not uncommon, particularly in some agricultural pursuits.[1, 2]

Early in the history of leptospiral investigations the rice fields were recognized to be endemic or epidemic foci of infections in Japan.[3] Subsequently the infection hazard in rice fields for agriculturists was demonstrated in other parts of Asia.[4, 5] Approximately 20 years ago, Mino[6] first drew attention to leptospiral infections in Italian rice fields. Thereafter, the annual endemic or epidemic outbreaks of this disease in Italian rice-fields workers were intensively studied.[2] In Italian rice fields, approximately 10,000 cases are estimated to occur annually.[2] In recent years rice-field leptospirosis has been recognized in other countries, notably in Spain and France where the cultivation of rice has been expanded.[7, 8, 9] To determine if analogous foci of leptospiral infections occur in Turkey, a serologic survey was conducted on man and domestic animals in the South Central portion of the country.

The available information on leptospirosis in Turkey is limited. Sporadic cases in man have been reported since 1915 when Riza and Nuzhet[10] reported a case based on clinical evidence; the first isolate, however, was obtained in 1922.[11] Bernkopf *et al.* in 1948[12] drew attention to the presence of antibodies against *Leptospira grippotyphosa* in Turkish cattle shipped into Israel. Leptospirosis was also observed in dogs and *L. canicola* was isolated in 1954.[13] More recently, cases of leptospirosis in cattle, sheep and goats have been demonstrated by cultural, histologic and serologic techniques.[14-17]

The present report shows a high prevalence of leptospiral antibodies in the human and livestock populations of rice-raising areas of South Central Turkey.

MATERIALS AND METHODS

Area under study. The area under study is located in Silician plains between the Tarus mountains and the Mediterranean Sea (Figure 1). This region was arbitrarily divided into four areas based on the number of years in rice production. Area I is the largest of the areas and contains a high percentage of arable land where many closely located villages are found. Rice production was relatively new and rice became an important crop only about 10 years ago when construction of a dam and canals for irrigation was completed.

In Area II rice has been extensively grown for the past 60 years. Area II is adjacent to Area I and is surrounded on the north by a low mountain range. The water for irrigation of the rice fields is supplied by tributaries of the Ceyhan River.

Area III is located approximately 100 miles northeast of Area I and II and is somewhat isolated by surrounding mountains. Rice has been grown in this region for more than 300 years. It has also been reported that rice was grown here 3,000 years ago during the period of the Hittite civilization. There would be little, if any, intermingling of livestock between this area and the other areas of study.

Area IV is a large demonstration and animal breeding farm operated by the federal govern-

* Major, USAF (VC), Veterinarian formerly assigned to Fourth Epidemiological Flight, U. S. Air Force, Izmir Turkey. Present address—6550th U. S. Air Force Hospital, Patrick Air Force Base, Florida.

† Division of Veterinary Medicine, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington 12, D. C.

‡ Peudik Bacteriological Institute, Istanbul, Turkey.

ment and devoted to the improvement of livestock, wheat, and to a lesser extent cotton and other crops; however, rice is not grown. The animal husbandry practices and facilities are better than those of the surrounding Silician plains. The contact among livestock on this farm at common watering ponds is considerably more restricted than in the neighboring villages.

In the entire region under study, rice is planted during April and the first half of May. The soil is very moist at this time as a result of the heavy rainfall each Spring. The fields are not flooded until June—6 to 8 weeks after planting. Initially the fields are flooded to a depth of approximately 10 centimeters. The fields are weeded once and sometimes twice in late June and/or July. The second weeding, if necessary, is completed just prior to increasing the depth of the water to 20 centimeters. The pH of the soil in this rice-growing area, as reported by the Faculty of Agriculture, University of Ankara (Personal communication), ranges from 6.2 to 8.0. The pH of water from tributaries of the Ceyhan River is 7.2. All phases of planting, weeding and harvesting, with few exceptions, are carried out by men, women and children of the local village. Few, if any, migratory farm workers are brought in from outside the rice-producing area, as is commonly practiced in Italy. At harvest time, September and October, the fields are no longer inundated; but many muddy patches and small pocket-like water-holes are usually present in most fields.

Animal husbandry practices for the various livestock species are similar for most villages. Cattle and water buffalo of each village are kept in a common herd and driven to graze on the open range each morning and returned to their respective owners in late afternoon. The sheep and goats may or may not be herded together, depending on the total number in the village. They are usually but not always returned to the village each evening, depending on the season of the year, the distance to range, and on grazing conditions. The relatively small numbers of horses and donkeys are used principally for riding and are usually kept near the village.

Collection of sera. Blood samples were collected from 240 human beings, 914 cattle, 115 horses, 109 goats, 50 donkeys and 49 sheep that were distributed in or about villages located in the four arbitrarily defined areas. Within each area, representative villages were selected by the district Agricultural and Veterinary office, without bias, as blood-collection sites. The blood samples were collected aseptically from 17 June 1957 to 11 July 1957. Collected samples were transported within 5 hours to a temporary laboratory set up at an air base near Adana where serums were processed and stored in a freezer. When collections were completed, the samples were packed in CO_2 ice and shipped to the Walter Reed Army Institute of Research (WRAIR), where they were maintained in a frozen state until the time of testing.

Serologic techniques. The basic serologic procedure employed in this survey was the microscopic-agglutination (agglutination-lysis) test of Schuffner and Mochtar.[18] This procedure has been slightly modified for use in this laboratory and is described in detail elsewhere.[19] Generally each serum sample was diluted 4-fold with phosphate-buffered saline solution (pH 7.4) to provide final serum dilutions after the addition of antigen of 1:100 through 1:6400. If necessary, additional dilutions were made to determine end-point reactions. Serums were screened with the following 18 live antigens, which consisted of 5- to 7-day-old cultures in Stuart's medium:

L. andamana, strain CH 11
L. semaranga, strain Veldrat S-173
L. celledoni, strain Celledoni
L. bataviae, strain Van Tienen
L. pomona, strain Pomona
L. djasiman, strain Djasiman
L. alexi, strain HS-616
L. grippotyphosa, strain Moskva V
L. borincana, strain HS-622
L. sentot, strain Sentot
L. autumnalis, strain *Fort Bragg*
L. ballum, strain Mus 127
L. canicola, strain Hond Utrecht IV
L. icterohemorrhagiae, strain Wijnberg
L. pyrogenes, strain Salinem
L. sejroe, strain M-84
L. javanica, strain Veldrat Bataviae 46
L. australis, strain Ballico

All of the human and 102 of the bovine serums were also tested with *L. mini*, strain Sari. In some instances (16), to elucidate the significance of reactions against *L. sejroe* and/or *L. borincana*, bovine samples were also tested against the other members of the *hebdomadis* serogroup. The 18 screening antigens cover the broad spectrum of

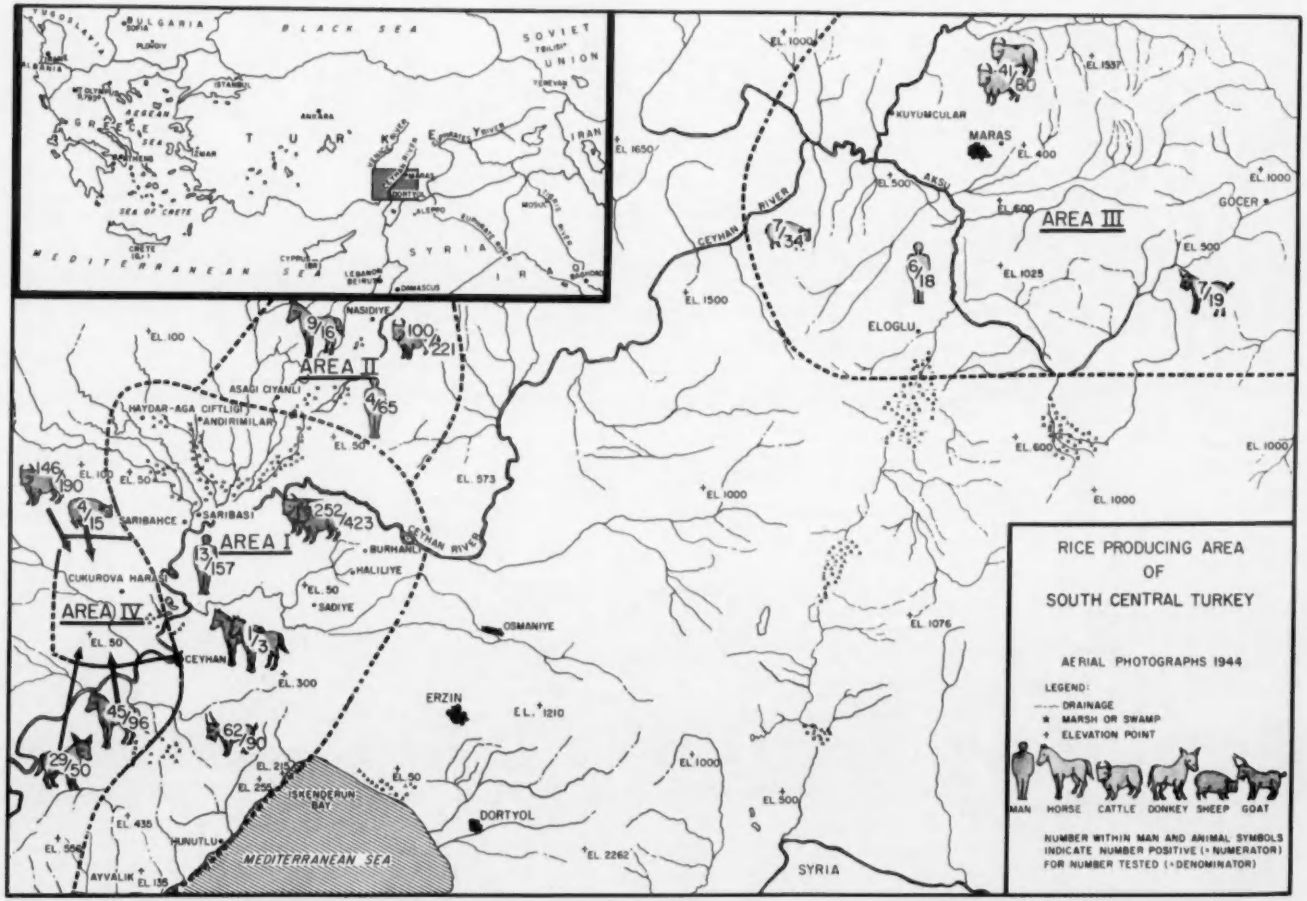


FIG. 1. Map of south central Turkey showing the prevalence of leptospiral agglutinins in various mammalian species located in three rice-producing areas (I, II, III) and in one animal breeding farm area (IV).

cross-reactions with recognized serotypes and also include these serotypes that have been prominently associated with rice-field leptospirosis.

Reactions at 1:100 dilutions and above, in which 75% or more of the cells were agglutinated or "lysed", were considered significant. "Partial" reactions (viz. 50 to 75% of the cells agglutinated) at the lowest serum dilution were noted but not included in the presented tables.

RESULTS

Prevalence of seropositive serums. The distribution of positive reactions observed in serums from different species in the three rice-raising areas and the one adjacent area is shown in Table 1. Employing the WRAIR criterion for a significant serologic reaction, the prevalence ratio of leptospiral agglutinins was found to be 9.6 for man, 59.0 for cattle, 63.3 for goats, 22.4 for sheep, 47.8 for horses and 58.0 for donkeys.

The reactions of serums of all species except horses were primarily obtained against *L. grippotyphosa* antigen or against antigens from members of the *hebdomadis* group, viz., *L. sejroe*, *L. borincana* and *L. mini*. Nineteen of the 23 unequivocal serologically positive reactions in

TABLE 1

Prevalence of leptospiral agglutinins in human beings and domestic animals in areas of south-central Turkey

Area	Species	No. tested	No. serums showing predominant reactions against			Total no. positive	Per cent positive
			Grippotyphosa	Sejroe*	Others†		
I	Man	157	11	1	1	13	8.3
	Cattle	423	84	128	40	252	59.6
	Goats	90	56	2	4	62	68.9
	Horses	3	0	0	1	1	33.3
II	Man	65	3	0	1	4	6.2
	Cattle	221	28	52	20	100	45.2
	Horses	16	4	0	5	9	56.2
III	Man	18	5	0	1	6	33.3
	Cattle	80	19	16	6	41	51.2
	Goats	19	6	0	1	7	36.8
	Sheep	34	6	0	1	7	20.6
IV	Cattle	190	8	132	6	146	76.8
	Sheep	15	0	2	2	4	26.7
	Horses	96	9	9	27	45	46.9
	Donkey	50	11	10	8	29	58.0
Totals	Man	240	19	1	3	23	9.6
	Cattle	914	139	328	72	539	59.0
	Goats	109	62	2	5	69	63.3
	Sheep	49	6	2	3	11	22.4
	Horses	115	13	9	33	55	47.8
	Donkeys	50	11	10	8	29	58.0

* *L. sejroe* and/or *L. borincana* or *L. mini*.

† Includes predominant titers to other serotypes and multiple reactors to same titer against 2 or more serotypes distributed within species as follows: Man—*L. autumnalis* 2, multiple 1; Cattle—*L. autumnalis* 6, *L. semaranga* 8, *L. andamana* 5, *L. batavia* 2, multiple 51 (grippotyphosa and sejroe 42); Goats—*L. autumnalis* 5; Sheep—*L. autumnalis* 2, *L. ballum* 1; Horses—*L. autumnalis* 13, *L. andamana* 3, *L. pyrogenes* 1, *L. australis* 3, multiple 13; Donkeys—*L. autumnalis* 4, multiple (grippotyphosa and sejroe 4).

human sera were elicited with *L. grippotyphosa* antigen. Some of the *L. grippotyphosa* reactors also showed co-agglutinins against *L. djasiman*, *L. australis* (Ballico) and *L. autumnalis*. The association of these cross-reactions with *L. grippotyphosa* titers is not uncommon and has been observed in other studies conducted in this laboratory. Two of the human samples showed predominant titers against *L. autumnalis*, one against members of the *hebdomadis* group and one cross-reacted with multiple antigens. The observed titers of 19 of the serums were at the level of 1:100, and 4 were at the level of 1:400 serum dilution.

The overall ratio of *L. grippotyphosa* and *hebdomadis* group reactors in cattle was considerably less disparate than that observed with human serums. Approximately 61% of the positive bovine serums showed predominant titers against members of the *hebdomadis* group and 26% against *L. grippotyphosa*. Agglutinin titers ranging from 1:100 to 1:25,600 were observed in both instances. A large percentage of cross-reactions between *L. grippotyphosa* and the *hebdomadis* group antigens was observed in serum samples showing predominant titers against the respective strains, and in 42 serums multiple reactors to the same titer against both groups were observed. Sixteen serums with predominant titers against the screening antigens in the *hebdomadis* group were also tested with 13 other *hebdomadis* group serotypes. No higher titers were obtained. Generally, highest titers were elicited with the *L. sejroe* antigen. Relatively few serums showed predominant titers against other serotypes; viz., *L. autumnalis*—6, *L. semaranga*—8, *L. andamana*—5 and *L. bataviae* —2, and these reactions were only observed at the initial serum dilution level of 1:100.

All but 7 of the 69 positive goat serums showed maximum titers against *L. grippotyphosa*. The titers ranged up to 1:25,600 and the majority of the reactions were at 1:1600 serum dilution levels and greater. Three serums had titers in the order of 1:100 to 1:400 against *hebdomadis* group antigens, and reactions with *L. autumnalis* ranging in titer from 1:100 to 1:1600 were seen in five samples. A large number of the *L. grippotyphosa* reactors showed co-agglutinins against *L. djasiman* and *L. autumnalis*, but cross-reactions with *hebdomadis* group antigens were rare.

Six of the 11 positive titers revealed in sheep serums were against *L. grippotyphosa* and ranged up to 1:1600. Here again no cross-reactions with antigens of the *hebdomadis* group were observed. The remaining positive serums showed reactions at a serum dilution level of 1:100 against *L. sejroe* (2), *L. autumnalis* (2) and *L. ballum* (1). The lowest percentage (22.4) of reactors among domestic animals was observed in sheep.

A greater variety of serologic reactions was observed in horses. Of the 55 reactors, 13 showed predominant titers against *L. grippotyphosa* at serum dilution levels up to 1:400, 9 against antigens in the *hebdomadis* group from 1:100 through 1:6400 and at serum dilution levels of 1:100, 13 against *L. autumnalis*, 3 against *L. andamana*, 1 against *L. pyrogenes*, 1 against *L. ballum* and 15 reactors to same titer against multiple unrelated antigens. Serums showing predominant titers against *L. grippotyphosa* and *hebdomadis* group antigens frequently contained co-agglutinins against the respective antigens as well as others. The multiplicity and diversity of cross-reactions in equines are not unusual and have been observed elsewhere.[20]

The ratio of *L. grippotyphosa* to *hebdomadis* group reactors among donkeys was 11 to 10. Four serums also reacted against *L. autumnalis* and four reacted to the same titer against *L. grippotyphosa* and the *hebdomadis* group antigens. The observed titers were in the order of 1:100 except for four samples with titers of 1:400 against antigens of the *hebdomadis* group.

It is noteworthy that although the serum titers in the human, goat and sheep samples from rice-raising areas were almost exclusively restricted to *L. grippotyphosa* antigen, this was not true for the cattle serums (Tables 2 and 3). Generally, in bovine serums the frequency of predominant reactions against *hebdomadis* group antigens was more than 2-fold greater than against *L. grippotyphosa*. However, within areas, there was considerable variation in the relative frequencies of reactions against the two different types of antigens (Table 2). In fact, in some villages, *e.g.*, Haliliye, Ayvalik and Gocer, the number of bovine *L. grippotyphosa* reactors exceeded the number of reactors against the *hebdomadis* group antigens.

The percentages of serologically positive reactions in domestic animals in the non-rice-raising area of Cukurova Horasi, were comparable to those observed in the rice areas. The

TABLE 2

Distribution of titers in bovine serums against L. grippotyphosa *and* hebdomadis *group antigens by collection areas*

Area	Village	Total no. tested	Distribution of titers against										Ratio grippo/ hebd reactors
			L. grippotyphosa					*Hebdomadis gp. antigens*					
			1:100	1:400	1:1600	>1:1600	total	1:100	1:400	1:1600	>1:1600	total	
I	Burhanli	49	1	2			3	21	6			27	0.11
	Sadiye	56	4	2			6	4	2			6	1.00
	Haliliye	71	10	7	1		18	8	6	1		15	1.20
	Saribasi	62	1	5	6		12	13	5	1		19	0.63
	Saribahee	29	4	2	1		7	6	3			9	0.78
	Haydar-Aga	59	4	1		1	6	9	16	4		29	0.21
	Andirimilar	36		3	4	9	16	7	6	2		15	1.07
	Ayvalik	40	2	9	3		14	3	1			4	3.50
	Hunutlu	21	1	1			2	4				4	0.50
	Total	423	27	32	15	10	84	75	45	8		128	0.66
II	Nesidiye	98	2		1		3	16	4			20	0.15
	Asagi-Ciyanli	123	8	10	4	3	25	24	8			32	0.78
	Total	221	10	10	5	3	28	40	12			52	0.54
III	Kuyumcular	41	3				3	2	4	2	1	9	0.33
	Gocer	20	1	4	1	9	15	1				1	15.00
	Eloglu	19	1				1	5	1			6	0.17
	Total	80	5	4	1	9	19	8	5	2	1	16	1.19
IV	Cukurova	190	4	3	1		8	52	71	9		132	0.06
	Total.....	914	46	49	22	22	139	175	133	19	1	328	0.42

relative number of seropositive cattle in this area was higher than that in the other three areas (Table 1). Furthermore, in contrast to findings in the rice-field areas, the number of bovine reactors to the *hebdomadis* group antigens was remarkably higher than to *L. grippotyphosa* (Table 2). This disproportionate ratio was not observed in the equine serums (Table 3).

Distribution of seropositives within areas. The differences in the percentage of serologically positive human sera between Areas I and II were relatively small; however, these percentages, 8.3 and 6.2 respectively, were considerably lower than the 33.3% reactors observed in Area III (Table 1). It is noteworthy that a considerably smaller number of samples were obtained from Area III in comparison to Areas I and II. In addition, the percentage of positive reactors within the 8 villages of Areas I and II, in which 15 or more human serums per village were collected, varied from 0 to 18. It is therefore difficult to relate the higher prevalence ratio of antibody in human beings to the length of time that rice had been cultivated in this area. More likely, it reflects a very local situation or a bias inadvertently introduced by the method of sampling.

Similarly, the distribution of positive reactions in serums from domestic animals could not be related to the duration of rice cultivation in the three areas. Considerable variation in the percentage of seropositives between villages was observed. For example, although the overall percentage of serologically positive bovine serums ranged from 45 to 59 in the three rice-raising areas (Table 1), the rate of seropositives between villages in Area I varied from approximately 27 to 94%, in Area II from 27 to 59%, in Area III from 36 to 80%. It was apparent from a tabu-

TABLE 3

Distribution of titers in goat, sheep and equine serums against L. grippotyphosa *and* hebdomadis *group antigens by collection areas*

Areas and hosts	Village	Total no. tested	Distribution of titers against										Ratio grippo/ hebd reactors
			L. grippotyphosa					*hebdomadis* group antigens					
			1:100	1:400	1:1600	>1:1600	total	1:100	1:400	1:1600	>1:1600	total	
Goats													
I	Ayvalik	66	5	9	24	18	56					0	56.00
	Hunutlu	24						2				2	0/2
III	Gocer	19		1	3	2	6					0	6/0
	Total	109	5	10	27	20	62	2				2	32.00
Sheep													
III	Kuyumcular	11					0					0	
	Gocer	23	1	3	2		6					0	6/0
IV	Cukurova	15					0	2				2	0/2
	Total	49	1	3	2		6	2				2	3.00
Horses													
I	Hunutlu	3					0	1				1	0/1
II	Nesidiye	9	1	1			2					0	2/0
	Asagi-Ciyanli	7	2				2					0	2/0
IV	Cukurova	96	7	2			9	3	4	1	1	9	1.00
	Total	115	10	3			13	4	4	1	1	10	1.30
Donkey													
IV	Cukurova	50	11				11	6	4			10	1.10

lation of the distribution of bovine serologic titers by villages (Table 2) that there were recent or current episodes of infection in some villages at the time the samples were collected. This was evident from the relatively high frequency of elevated titers in the order of 1:1600 and above, and was particularly noteworthy for *L. grippotyphosa* reactors in Andirimilar and Saribasi (Area I), in Asagi-Ciyanli (Area II) and Gocer (Area III) and to a lesser extent in Ayvalik (Area I). Recent occurrence of infection in cattle with a member of the *hebdomadis* group was also indicated in Haydar-Aga (Area I) and Kuyumcular (Area III).

The serum samples from goats, sheep and horses were derived from relatively few villages and not all areas were represented by the respective species. Consequently, a comparison of findings in each area for the respective species is not possible. The distribution of titers from these domestic animals in the villages where collections were possible is shown in Table 3. It is interesting to note that in Ayvalik (Area I) and Gocer (Area II) a high percentage of goats showed evidence of recent or current infection with a member of the *grippotyphosa* group. In these same areas high titers were also noted in sheep (Table 3) and bovine (Table 2) serums.

Prevalence of seropositive reactions in human and animal serums according to sex and age. All except 30 of the 240 human serums were obtained from males. Local mores precluded selection of a more representative number of blood specimens from females. No significant differences in prevalence ratios attributable to sex were observed within the limitation of the relative numbers of male and female samples. Ten and 9.6% of the serums from females and males, respectively, were positive at titers of 1:100 and greater.

The range in age of the human beings tested was generally 12 to 60 years; relatively few samples were obtained from persons over 60 years old. The distribution of serums by age, grouped at either 5 to 10 year intervals, did not reveal a stepwise increase in the percentage of positive reactions with increased age, although the serums were fairly evenly distributed among age groups. The lowest rates of seropositives, viz., 6.5% and 4.9%, were in the youngest (11 to 20 years) and oldest (over 50 years) age groups respectively. In the intermediate age groups the percentage of positives varied from 7.3 to 14.0.

Only 78 of the 914 serums from cattle were obtained from males; 46% of these were positive in contrast to 60% seropositives obtained from females. This difference in prevalence ratios reflected the fact that a large proportion of the specimens from males, approximately 30%, were obtained from Nesidiye and Hunutlu, villages where a relatively small number of cattle showed positive reactions.

The cattle tested ranged up to 10 years in age with good age distribution of animals (over 50 samples) for each year up to 9 years old. Significant titers of 1:100 or greater were observed in 44% of the cattle that were 1 year old or younger. In successive yearly age groups the percentages of seropositives were notably higher, ranging from 53 to 69%, but could not be correlated with age. High titer reactions of 1:1600 or greater, indications of recent or current infections, were observed in approximately 6% of the animals over 5 years of age.

The goat serums were derived, with few exceptions, from 2- to 4-year-old females. Five samples were obtained from younger or older animals and several males were represented in this series. The successive rates of seropositive reactions (titers of 1:100 or greater) among the 17, 52 and 35 samples from the 2-, 3- and 4-year-old age groups, respectively, were 23%, 67% and 77%. The titers of serums from 2-year-old goats were in the order of 1:100 to 1:400, and all but one of these samples were obtained from Hunutlu. Almost all of the specimens from 3- and 4-year-old goats were obtained from either Ayvalik or Gocer where recent outbreaks of infection were indicated by the large number of high-titer serums (Table 3). Consequently the markedly lower prevalence ratio in the 2-year-old goats could not be attributed to age.

All of the sheep blood specimens were obtained from females, ranging generally from 2 to 6 years in age. In this relatively small number of samples no correlation of seropositive ratios with age was observed.

The equine samples, with the exception of 19 horses, were obtained from mares. The age of the animals sampled ranged from 2 to 17 years and more than 75% of both horses and donkeys were 7 or more years old. The highest percentage of reactors was in the oldest age group of donkeys and horses. Generally, correspondingly lower percentages of seropositives were observed in younger age groups.

DISCUSSION

The observations in this study indicate that the rice-field regions of south central Turkey are epidemic or endemic foci of leptospiral infections for man and animals. These findings were not unexpected in view of the history of leptospiral infections associated with the raising of rice. However, some features of leptospirosis in Turkey appear to differ from those seen in the rice-field areas of other Mediterranean countries. These differences relate primarily to the prevalence and types of antibodies found.

The determination of infecting serotype on the basis of the host serologic response is subject to important limitations and only provides presumptive evidence of infection with specific leptospiral types.[21] The presumptive evidence in this study points to *L. grippotyphosa* and one of the serotypes of the *hebdomadis* group, possibly *L. sejroe*, as the predominant types found in this area. In this regard, these findings are more reminiscent of the Field Fever infections found in Eastern European farming communities[22, 23] than the *L. bataviae* and *L. icterohemorrhagiae* infections predominating in Italian rice fields or the *L. icterohemorrhagiae* and *L. ballum* infections in rice fields of Spain and France. *L. grippotyphosa* and serotypes in the *hebdomadis* group, viz., *L. sejroe*, *L. saxkoebing* and *L. mini*, do, however, share a minor role in the etiology or rice-field leptospirosis in Italy.[2] Generally, the occurrence of infections with various serotypes is related to the presence and density of the specific rodent hosts which serve as major carriers of select serotypes.

In contrast to the attention directed to human cases of leptospirosis in the rice fields of southern Europe, leptospirosis in rice-field workers in

Turkey has not been particularly noted. The clinical unawareness of this disease is not unusual in the history of leptospirosis. More frequently the infections are mild and exhibit protean manifestations characteristic of numerous other diseases. Furthermore, the outbreaks of this disease in European rice fields are prominent in migrant workers newly engaged in rice production, or in new areas converted to the growth of rice. In the region studied, the shortest length of time that rice had been in cultivation was approximately 12 years—a sufficient length of time to stabilize the disease situation. Moreover, the cultivation of rice is managed on a local level without outside help. Consequently the number of new susceptibles may be governed by local birth rates.

The prevalence ratio of leptospiral antibodies in human beings (9.6%) was considerably lower than the percentage of seropositives reported for rice areas of other countries. However, this percentage was based on a very conservative criterion for a positive reaction, viz., 75% or more of cells agglutinated or "lysed" at serum dilutions of 1:100 or higher. However, if the "partial" reactions (50 to 75% of the cells agglutinated) at 1:100 serum dilution had been tabulated with the seropositives, the prevalence of reactors could have been increased to 32%—a ratio more comparable with findings in other rice-field regions.[24, 25] The stepwise increase in percentage of seropositives with increased age noted in other rice-field surveys was not observed in this study. It is highly probable that infections are incurred at an early age and that, in subsequent years, residual antibody titers decrease below detectable levels.[2]

The relatively rare occurrence in human, goat, and sheep serums of antibodies against antigens of the *hebdomadis* group was particularly notable in view of the serologic findings in cattle. In the three rice-field areas the number of bovine samples with predominant titers against members of the *hebdomadis* group was either equal to, or 34 to 46% greater than, the *L. grippotyphosa* reactors. However, in the non-rice-raising regions of Cukurova nearly all of the serologic reactions in cattle were restricted to members of the *hebdomadis* group. Moreover, the overall percentage of bovine seropositives exceeded those in the rice areas by 30 to 70% (Table 1). Apparently the natural foci of infections with members of the *hebdomadis* group were not necessarily confined to rice fields.

It was evident from the distribution of serologic titers by villages (Tables 2 and 3) that episodes of infection in domestic animals had recently occurred or were in progress in certain villages at the time collections were made. In fact, in one of the villages, Gocer, the local report of an outbreak of disease in cattle, characterized by abortion, hemoglobinuria and loss of weight, in retrospect is significant in view of subsequent serologic findings. Signs of disease in goats and sheep in this village were not reported, although titers comparable with those of cattle were observed. Unfortunately, no human blood samples were offered in Gocer. It is also interesting to note that many of the high-titer reactions—attributable to recent or current infections—were observed in cattle that were over 5 years of age. In view of the high percentage of positive serums from cattle up to 1 year of age, it is not unlikely that some of these animals may have had a repeated infection.

The presence of leptospirosis in livestock is obviously significant in livestock sanitation and may be important from a public health viewpoint. The primary hosts of *L. grippotyphosa* are rodents, principally members of the genus *Microtus*; however, livestock can be infected and serve as secondary reservoirs. In Israel and elsewhere, numerous human infections are attributable to direct or indirect contact with infected cattle.[26] According to M. Tolunay of Ankara University and H. W. Setzer of the U. S. National Museum (personal communications) the rodents known to be present in the study area include *Microtus guntheri* and *Microtus arvalis*, which are known to carry *L. grippotyphosa* infection, and *Apodemus sylvaticus* and *Mus musculus*, which carry and shed organisms of the *hebdomadis* group. The relative roles of the rodents and livestock in the spread of leptospirosis in this area in relation to the cultivation of rice and other agricultural pursuits require further studies.

SUMMARY

A serologic survey for leptospiral agglutinins was conducted on serums from 240 human beings, 914 cattle, 115 horses, 109 goats, 50 donkeys and 49 sheep, located in three rice-raising areas and one adjacent livestock breeding area in south central Turkey. Employing a very conservative test criterion for a significant serologic

reaction, the percentages of positives in the various hosts were the following: man—9.6, cattle—59.0, goats—69.3, sheep—22.4, horses—47.8 and donkeys—58.0. Most of the predominant serologically positive reactions in human beings, goats and sheep were elicited with *L. grippotyphosa* antigen, a lesser number with screening antigens in the *hebdomadis* group. In bovine serums, however, in addition to the presence of agglutinins against *L. grippotyphosa*, a large proportion of seropositive reactions was primarily against antigens of the *hebdomadis* group, notably *L. sejroe*. The highest percentage of bovine seropositives was derived from the non-rice-raising area and most of the animals were reactors against the *hebdomadis* group antigens. Equine positive serums showed a large proportion of predominant titers against *L. grippotyphosa* and *hebdomadis* group antigens and also against *L. autumnalis*; however, a greater variety and multiplicity of agglutinins were observed in these serums.

The titers of human seropositives were either 1:100 or 1:400; however, titers observed in animal serums ranged up to 1:25,600. It was apparent from the distribution of titers that recent episodes of infection in livestock had occurred or were in progress in some of the villages at the time serums were collected.

Differences in prevalence ratios attributed to sex were not evident from the available data for any of the species tested. Generally, the percentage of positives was lowest in youngest age groups of the various species; however, a stepwise increase in the percentage positive in successively older groups was not noted.

These findings indicate that the rice-field regions of south central Turkey are epidemic or endemic foci of leptospiral infections in man and domestic animals, and that most of these infections are presumably caused by *L. grippotyphosa* and a member of the *hebdomadis* group, possibly *L. sejroe*.

ACKNOWLEDGMENTS

The authors are grateful to 1/Lt Leslie B. Davenport, Jr., USAF (MSC) Medical Entomologist, 4th Epidemiologist Flight; to Drs. Vefik Bora and Feyyaz Salman, district veterinary and health officers, respectively, in Ceyhan; Drs. Fehmi Caldere and Alaettin Cetin, provincial Veterinary Directors in Adana and Maras; and Mr. Mursel, U. S. Director of the Cukurova Livestock Farm, who made possible the collection of serums. The authors are also indebted to Mrs. F. Seckel, Medical Audio Visual Department, Walter Reed Army Institute of Research for the drawing of the map of Turkey.

REFERENCES

1. Alston, J. M., and Broom, J. C., 1958. *Leptospirosis in Man and Animals*. E. S. Livingstone Lt'd., Edinburgh.
2. Babudieri, B., 1957. Leptospirae and leptospirosis in Italy. *Sc. med. ital.* **5:** 658–702.
3. Tohyama, Y., 1924. Experimental studies and actual test results concerning prophylaxis of Weil's disease (Spirochaetosis icterohaemorrhagica) agriculturally viewed. *Japan Med. World*, **4:** 193–198.
4. Taylor, J., and Goyle, A. N., 1931. Leptospirosis in the Andamans. *Indian M. Res. Mem.*, no. 20: 1–190.
5. Vaucel, M., 1938. Leptospiroses en Indochine Française. Types serologiques observés. *Acta Convent. tertii de trop. atque malar. morbis*, Amsterdam, **1:** 372–380.
6. Mino, P., 1939. Ueber Leptospirosen bei den Arbeitern der Reisfelder Oberitaliens. *Ztschr. Immunitätsforsch.*, **96:** 466–485.
7. Covaleda, J., Pumarola, A., and Cantarell, J., 1953. Leptospirosis por *L. ballum* en los trabajadores de arrozal de la region de Camarles (Delta del Ebro). *Rev. Iber. Parasitol.*, **13:** 289–298.
8. Babudieri, B., 1955. El problema de las leptospirosis. *Rev. san. e hig. púb.*, **29:** 135–152.
9. Picard, J., 1956. La pathologie des rizieres de la Camargue. *Rev. path. gen. comparée*, **56:** 141–150.
10. Riza, R., and Nuzhet, A., 1915. Morbus Weil cases. *Sihhiye mecmuasi*, **3:** 974–993. Cited by Hakioglu.[17]
11. Serif, H., 1922. The morbus Weil cases in Istanbul. *Istanbul seririyati*, **2:** 101. Cited by Hakioglu.[17]
12. Bernkopf, H., Stuczynski, L. A., Gotlieb, T., and Halevi, C., 1948. Serological examination of human and cattle sera from Palestine for the presence of antibodies against a bovine strain of leptospira. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **42:** 259–265.
13. Unat, K., and Gürtürk, S., 1954. Türkiye'de *Leptospira canicola* infeksiyonu. *Türk vet. hekim. dern. derg.*, **24:** 1705–1708.
14. Ozgen, H., and Tunis, M., 1954. Türkiye'de ilk olarak *Leptospira bovis* susunum kulturel gelistirilmesi. *Türk vet. hekim. dern. derg.*, **24:** 1865–1866.
15. Hakioglu, F., 1955. Ictero-hemoglobinurie enzootique chez les bovides en Turquie causee par un leptospire du genre de *L. bovis*. VII[e] *Congr. internat. path. comparée*, Lausanne, 26–31 May, 1955, **2:** 196–197.
16. Hakioglu, F., 1956. Zum erstenmal durch histologische Untersuchung festgestellte Ziegen-und-Schaf-Leptospirosis in der Turkei. *Zentralbl. allg. Path.*, **95:** 525–527.
17. Hakioglu, F., 1958. Die in der Turkei durch

Leptospira bovis verursachte Rinderleptospirosis und die dabei beobachten Symptome sowie die festgestellten Hautveränderungen. *Deutsche Tierärtzl. Wchnschr.*, **65**: 404–406.

18. SCHÜFFNER, W., AND MOCHTAR, A., 1927. Versuche zur Aufteilung von Leptospirenstämmen, mit einleitenden Bemerkungen über den Verlauf von Agglutination und Lysis. *Centralbl. Bakt., Abt.* 1 *Orig.*, **101**: 405–413.
19. GOCHENOUR, W. S. JR., YAGER, R. H., WETMORE, P. W., AND HIGHTOWER, J. A., 1953. Laboratory diagnosis of leptospirosis. *Am. J. Pub. Health*, **43**: 405–410.
20. VAN DER HOEDEN, J., 1958. Epizootiology of leptospirosis. *Advances Vet. Sc.*, **4**: 277–339.
21. ALEXANDER, A. D., EVANS, L. B., TOUSSAINT, A. J., MARCHWICKI, R. H., AND MCCRUMB, F. R. JR., 1957. Leptospirosis in Malaya II. Antigenic analysis of 110 leptospiral strains and other serologic studies. *Am. J. Trop. Med. & Hyg.*, **6**: 871–889.
22. VAN THIEL, P. H., 1948. *The Leptospiroses*. Universitaire Pers Leiden.
23. RIMPAU, W., 1950. *Die Leptospirose. Eine kurze Darstellung für Arzte, Tierärzte und Gesundheitsbehörden*. Urban & Schwarzenberg, München.
24. BABUDIERI, B., 1944. Ricerche serologiche sulla frequenza delle infezioni da leptospire in risaia. *Rendic. ist. super. san.*, **7**: 537–547.
25. BABUDIERI, B., BARRERA, M., ALTIVA, V., GIL, P., VILLALONGA, I., AND MARIN, C., 1955. Estudio serologico de 253 trabajadores de los arrozales de Castellón de la plana frente a catorce tipos de leptospiras. *Rev. san. e. hig. púb.*, **29**: 161–166.
26. VAN DER HOEDEN, J., 1955. The epidemiology and epizootiology of leptospirosis in Israel. *J. Trop. Med. & Hyg.*, **58**: 202–204.

DISTRIBUTION OF EGGS OF DIFFERENT GEOGRAPHIC STRAINS OF *SCHISTOSOMA JAPONICUM* IN THE VISCERA OF INFECTED HAMSTERS AND MICE*

H. F. HSÜ AND S. Y. LI HSÜ

Department of Hygiene and Preventive Medicine, College of Medicine, State University of Iowa, Iowa City, Iowa

The distribution of eggs of the Formosan and Japanese strains of *Schistosoma japonicum* in the viscera of infected hamsters has been studied by Moore and Sandground.[1] They found that about 50 per cent of the eggs produced by this parasite were located in the walls of the large intestine and that there was essentially no difference between the two strains with respect to the distribution of eggs in the viscera.

Since the essential role in the pathogenesis of Schistosomiasis japonica has been ascribed to the parasite's eggs,[2] if Moore and Sandground's findings could be held true, the distribution of the pathological lesions would have shown no significant difference in the susceptible hosts infected with different geographic strains. Faust and Meleney,[3] while they were working in China, found that although in human infection with *S. japonicum* the colon was the part most involved, in all of their experimental dogs infected with the cercariae of this parasite the jejunum was the portion of the alimentary tract most intensely involved. Vogel and Minning[4] used the cercariae from *Oncomelania hupensis* from Kashing, China, to infect rhesus monkeys in Hamburg, Germany; they reported that the intestinal lesions were mainly in the large intestine. In Japan, Ito[5] found that in infected rabbits and mice, numerous egg lesions were found in the lower duodenum and upper jejunum, while egg lesions were fewer in the other parts of the intestines. The results of these three reports seem to indicate that the distribution of lesions of schistosomiasis japonica in the susceptible hosts varied with the geographic strains of the infective agent as well as with the species of the hosts.

* This investigation was supported by a research grant E-939 (C4), P. T. M. (5), National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, United States Public Health Service, and by a research grant from Abbott Laboratories, North Chicago, Illinois.

For further elucidation of this intricate problem, studies on the distribution of eggs of various geographic strains of this parasite in the viscera of various experimentally infected hosts seem necessary.

MATERIALS AND METHODS

Four geographic strains of *S. japonicum* were used: the Chinese, Formosan, Japanese, and Philippine. The cercariae of the Chinese strain were obtained from *Oncomelania hupensis* originating in Kashing, Chekiang, China (Vogel's strain); the Formosan, from *O. formosana* obtained from Changhua, Taiwan, China; the Japanese, from *O. nosophora* obtained from Kofu, Japan; and the Philippine, from *O. quadrasi*, obtained from Leyte, the Philippines. The sex of cercariae shed from an infected snail was determined by a previous inoculation into a mouse. Only those snails which were shedding one sex of cercariae were used. The cercariae shed from a snail were transferred with a small hair loop to a drop of water on a cover glass. The cercariae on the cover glass were counted and the number was controlled by careful manipulation. Even a single cercaria could be isolated in this way. The cover glass was then turned over. With the cercaria-containing surface directly in contact with the clipped moistened skin, the cover glass was placed on the abdomen of the experimental animal for inoculation.

Hamsters of 70 to 80 grams and albino mice of 16 to 20 grams were used as experimental animals. Each animal was infected by the cutaneous route with 2 or 100 cercariae with sexes equally divided. Exposure to 2 cercariae produced patent infections in 32 to 61% of the trials. The infected animals were killed on the 5th day after their stools were found positive with mature eggs. When an animal was killed, 11 internal organs—lungs, liver, spleen, stomach, duodenum, jejunum, ileum, caecum, ascending

colon, transverse colon, and descending colon (including rectum)—were isolated. The contents in the digestive tract were washed out before the different portions were divided. Each isolated organ was first digested in a flask containing digestive solution (1% pepsin, 0.5% HCl made up in 0.85% NaCl solution). After digestion, the contents of the flask were transferred to a 15-ml graduated conical tube and centrifuged. The supernatant fluid was then decanted and the tube was refilled with a 2% solution of KOH up to the 10 ml mark. The sediment in the centrifuge tube was then thoroughly mixed with the KOH solution to remove the remaining tissues and to free all the eggs from the rest of the sediment. Three samples of 0.1 ml were withdrawn by means of an Ives dropping pipette. Each sample was placed on a slide under a coverglass. All the eggs in each sample were counted under a microscope. The results of the three counts were averaged and multiplied by the diluting factor 100 to determine the number of eggs in the organ. If the count was below 10 eggs in the 0.1 ml sample, the contents in the centrifuge tube were centrifuged again. After the supernatant fluid was decanted, the eggs in the whole sediment were counted.

Throughout this paper, the number of eggs found in the respective organs of an infected animal will be given only as the percentage of the total number of eggs obtained from the 11 visceral organs. The duodenum, jejunum, and ileum were treated either separately as different organs or collectively as the small intestine; and the caecum, ascending colon, transverse colon, and descending colon were considered also either separately as different organs or collectively as the large intestine.

The statistical analyses used in this paper were mainly significance tests for the means. For the comparison of means of samples from two strains, the homogeneity of the variances was investigated by the *F*-test. If the variances were homogeneous, the Student's *t*-test was then performed. If the variances were heterogeneous, the modified procedure developed by Welch and Aspin[6, 7] was used. For the comparison of means of samples from four strains, the homogeneity of variances was tested by the Bartlett's method. If the variances were homogeneous, analysis of variance was made by the *F*-test. Pairwise comparisons of the means by the Student's *t*-test were followed if the general significance test pointed to real differences among the four means. If the variances were heterogeneous, the Kruskal-Wallis' one-way analysis of variance by rank[8] was performed. When real differences in variances among the four strains were found, the means of any pair of the four strains were again tested for the significance of difference by the modified procedure of Welch and Aspin.

Whenever abbreviations are used in the text, C means Chinese; F, Formosan; J, Japanese; and P, Philippine. In the tables, NS means "not significant" and S. D., "standard deviation."

RESULTS

(1) *Strain of* S. Japonicum *as a factor influencing the distribution of eggs in the host*

In hamsters infected with 100 cercariae of the F and J strains. The distributions of eggs of *S. japonicum* in the viscera of 9 hamsters infected with the F strain and of 12 with the J strain were studied. The mean of the total number of eggs obtained from the viscera of each of 9 hamsters infected with the F strain was 496,083; and of 12 infected with the J strain, 1,530,349. The standard deviation was 354,725 for the F strain and 441,378 for the J strain. The results of statistical analysis showed that the two means were significantly different at the 0.1 per cent level.

The numbers of eggs found in each of six organs (lungs, liver, spleen, stomach, small intestine, and large intestine) of a given animal, as mentioned above, are expressed as percentages of the total number of eggs found in that animal. The means and standard deviations of these percentages for the 9 animals infected with F strain as well as those for the 12 animals infected with the J strain are reported in Table 1. Concerning these means, it is interesting to note that with the F strain, the mean of percentages of eggs was 40.31 in the liver and 16.03 in the small intestine; whereas, with the J strain, these means were 14.99 in the liver and 46.24 in the small intestine. The results of statistical analyses showed that the difference between the means of the percentages of eggs in livers was significant at the 0.1 per cent level, and so was the difference between the means of the percentages of eggs in the small intestines. This holds, furthermore, generally

TABLE 1

Means and standard deviations of the percentages of eggs of S. japonicum *in different organs of hamsters infected with 100 cercariae of either the F or the J strain*

Organs	Means and standard deviations of the percentages of the eggs				
	F strain (N = 9)		J strain (N = 12)		Significance level of difference between the means
	Mean	S.D.	Mean	S.D.	
Lungs	0.04	0.04	0.09	0.20	NS
Liver	40.31	8.05	14.99	3.02	<0.001
Spleen	0.02	0.04	0.01	0.04	NS
Stomach	0.35	0.24	1.24	1.18	<0.05
S. intestine	16.03	8.28	46.24	14.76	<0.001
L. intestine	43.24	11.79	37.42	15.10	NS
Total	99.99		99.99		

true even when the three portions of the small intestine—duodenum, jejunum, and ileum—were considered separately.

The results of this study show that the distribution of eggs of *S. japonicum* in the visceral organs was different in the hamsters infected with the F and J strains.

In mice infected with 100 cercariae of the 4 geographic strains. As the distributions of the percentages of eggs of *S. japonicum* in certain visceral organs of hamsters were significantly different in the infections of the F and J strains, it was important to learn whether such difference would be found in other host-species. The albino mouse was used for an additional host-species for this study. Eleven mice were infected with the C strain; 12 with the F strain; 12 with the J strain; and 17 with the P strain. Each mouse was infected with 50 male and 50 female cercariae. The mean of the total number of eggs obtained from mice infected with the C strain was 626,626; with the F strain, 796,584; with the J strain, 784,115; and with the P strain, 1,108,517. The standard deviation for the C strain was 163,344; for the F strain, 320,793; for the J strain, 189,177; and for the P strain, 195,509. The results of statistical analyses showed that the means differed significantly at the 0.1 percent level between the P and the other three strains.

Following the pattern of the analysis used with the hamsters, the means of the percentages of eggs of this parasite in different visceral organs of the mice infected with the four strains are given in Table 2. The distributions of the percentages of eggs in the livers and small intestines were again of special interest. With the C and F strains, the means of the percentages of eggs in the liver were 42.77 and 43.00 respectively whereas with the J and P strains, 11.29 and 20.55 respectively. On the other hand, with the C and F strains, the means of the percentages of eggs in the small intestine were 33.76 and 40.47 respectively; while with the J and P strains, 81.27 and 61.91 respectively. The means of the percentages of eggs in the large intestine can be arranged in the following order: 22.9 for the C strain, 17.10 for the P strain, 13.77 for the F strain, and 7.20 for the J strain. The means of the percentages of eggs in the spleen and stomach

TABLE 2

Means and standard deviations of the percentages of eggs of S. japonicum *in different visceral organs of mice infected with 100 cercariae of the four different geographic strains*

Organs	Means and standard deviations of the percentages of eggs							
	C strain (N = 11)		F strain (N = 12)		J strain (N = 12)		P strain (N = 17)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Lungs	0.15	0.23	0.04	0.05	0.001	0.00	0.02	0.04
Liver	42.77	7.80	43.00	7.18	11.29	4.52	20.55	4.83
Spleen	0.18	0.32	1.78	1.34	0.12	0.27	0.13	0.36
Stomach	0.24	0.29	0.94	1.00	0.11	0.17	0.29	0.60
S. intestine	33.76	12.32	40.47	8.30	81.27	5.84	61.91	6.98
L. intestine	22.90	6.49	13.77	6.61	7.20	4.46	17.10	4.16
Total	100.00		100.00		99.99		100.00	

are also worthy of mention. In the spleen, the mean of the percentages of eggs for the F strain was 1.78, and only 0.18 or less for the C, J, and P strain. In the stomach, the mean of the percentages of eggs for the F strain was 0.94 and only 0.29 or less for the C, J, and P strains. The results of statistical analyses indicate that the above mentioned differences between the means were mostly significant at the 5 or 0.1 per cent level (Table 3).

In conclusion, we can say that in mice, the C and F strains of *S. japonicum* were characterized by a higher percentage of eggs in the liver and lower percentage of eggs in the small intestine. The C and F strains can be differentiated by the fact that in the C strain, the percentage of eggs in the large intestine was higher, and in the spleen and stomach lower, than in the F strain. The J and P strains can be differentiated by the fact that in the J strain the percentage of eggs in the small intestine was higher, and in the liver and large intestine lower, than in the P strain. The F strain is further characterized by its highest percentage of eggs in the spleen and stomach, and the J strain by the highest percentage of eggs in the duodenum and jejunum and lowest percentage of eggs in the caecum. The results of this study indicate that the percentage distribution of eggs of *S. japonicum* in the host's visceral organs also differs in mice infected with the four strains of the parasite.

In mice infected with 2 cercariae of the 4 geographic strains. Although the distributions of eggs of *S. japonicum* in the visceral organs of mice have been shown to be distinct in different geographic strains, it is of interest to know whether the distribution of eggs in the visceral organs will be affected by the intensity of infection of the parasite. As results of distributions of eggs of the four strains have been obtained in mice infected with 100 cercariae, the present series of experiments was made also with mice which were, on the contrary, infected with the minimum number of parasites, one male and one female.

Twenty mice were infected with the C strain, 21 with the F strain, 30 with the J strain, and 30 with the P strain. The mean of the total number of eggs obtained from mice infected with the C strain was 48,061; with the F strain, 58,834; with the J strain, 46,047; and with the P strain, 47,899. The standard deviation was 16,551 for the C strain, 15,298 for the F strain, 13,780 for the J strain, and 12,173 for the P strain. The F strain evidently produced more eggs than the other strains. The results of statistical analyses showed that the means differed significantly at the 1 per cent level between the F and J strains and also between the F and P strains, and at the 2 per cent level between the C and F strains.

Again the means of the percentages of eggs in different visceral organs of mice infected with two cercariae were studied (Table 4). In the liver, the C and F strains had higher means of the percentages of eggs than the J and P strains. In the stomach, the F strain had a higher mean of the percentages of eggs than did the other three strains. In the small intestine, the J strain had a higher mean of the percentages of eggs than did all the other strains. In the large intestine, the C and P strains had higher means of the percentages of eggs than the F and J strains. The P strain was further characterized by a higher mean of the percentages of eggs in the descending colon than all the other strains. The

TABLE 3

Results of the statistical tests comparing the means of the percentages of eggs in different visceral organs of mice infected with 100 cercariae of one strain with the corresponding means of the other strains

Organs	Significance level: comparison between strains					
	C vs F	C vs J	C vs P	F vs J	F vs P	J vs P
Lungs	NS	NS	NS	0.02	NS	NS
Liver	NS	<0.001	<0.001	<0.001	<0.001	<0.001
Spleen	<0.01	NS	NS	NS	<0.01	NS
Stomach	<0.02	NS	NS	<0.05	NS	NS
S. intestine	NS	<0.001	<0.001	<0.001	<0.001	<0.001
L. intestine	<0.001	<0.001	<0.02	<0.01	NS	<0.001

results of statistical analyses showed that the above mentioned differences were significant either at the 5 or at the 0.1 per cent level (Table 5). The strain differences in the distributions of eggs in the host are thus shown in the mice even when infected with only one male and one female schistosome.

(2) *Host-species as a Factor Influencing the Distributions of Eggs of* S. Japonicum

The results of the above studies have shown that either in the hamster or in the mouse, the distributions of eggs of *S. japonicum* in the visceral organs were different when either of them was infected with different strains of the parasite. It is important to know whether the type of distribution of eggs in the visceral organs in the hamster and mouse will in general be the same, if both of them are infected with the same strain of the parasite. The data of the egg distribution obtained above from the 9 hamsters and 12 mice infected with 100 cercariae of the F strain, and from the 12 hamsters and 12 mice infected with 100 cercariae of the J strain were re-analyzed for this purpose.

The mean of eggs found in the visceral organs of hamsters infected with the F strain was 496,083; and of the mice, 796,584. The standard deviation was 354,725 for the eggs from the hamsters and 320,793 for the eggs from the mice. The results of statistical analyses showed that the difference between the means was not significant. The mean of the eggs found in the visceral organs of the hamsters infected with the J strain was 1,530,349; of the mice, 784,115. The standard deviation was 441,378 for the eggs from the hamsters and 189,177 for the eggs from the mice. The results of statistical analyses showed that the difference between these two means was significant at the 1 per cent level.

TABLE 4

Means and standard deviations of the percentages of eggs of S. japonicum *in different visceral organs of mice infected with two cercariae (1 male and 1 female) of the four different geographic strains*

Organs	Means and standard deviations of the percentages of eggs							
	C strain (N = 20)		F strain (N = 21)		J strain (N = 30)		P strain (N = 30)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Lungs	0.002	0.004	0.006	0.02	0.19	0.99	0.05	0.13
Liver	42.36	6.07	54.06	17.22	25.42	2.08	28.89	10.38
Spleen	0.001	0.01	0.08	0.24	0.01	0.04	0.004	0.01
Stomach	0.02	0.04	0.50	0.65	0.01	0.06	0.01	0.04
S. intestine	11.75	12.14	16.45	12.26	43.06	27.94	12.67	23.43
L. intestine	45.87	19.38	28.89	15.20	31.31	24.88	58.37	25.65
Total	100.00		99.99		100.00		99.99	

TABLE 5

Results of the statistical tests comparing the means of the percentages of eggs in different visceral organs of mice infected with two cercariae of one strain with the corresponding means of the other strains

	Significance level: comparison between strains					
	C vs F	C vs J	C vs P	F vs J	F vs P	J vs P
Lungs	NS	NS	NS	NS	NS	NS
Liver	<0.05	<0.001	0.01	<0.001	<0.001	NS
Spleen	NS	NS	NS	NS	NS	NS
Stomach	<0.01	NS	NS	<0.01	<0.01	NS
S. intestine	NS	<0.001	NS	<0.001	NS	<0.001
L. intestine	<0.02	<0.05	NS	NS	<0.001	<0.001

Concerning the means of the percentages of eggs in different visceral organs of hamsters and mice, the results of statistical analyses are shown in Table 6. With the F strain, the mean of the percentages of eggs in the small intestine was 16.03 for the hamster and 40.47 for the mouse; whereas, in the large intestine it was 43.24 for the hamster and 13.77 for the mouse. This indicates that with the F strain, the mean of the percentages of eggs in the small intestine was lower in the hamster than in the mouse, and vice versa in the large intestine. With the J strain, the mean of the percentages of eggs in the small intestine was 46.24 for the hamster and 81.27 for the mouse; whereas, in the large intestine it was 37.42 for the hamster and 7.20 for the mouse. Accordingly with the J strain the mean of the percentages of eggs in the small intestine, as with the F strain, was also lower in the hamster than in the mouse, and vice versa in the large intestine. The results of statistical analyses indicate that the differences between the means of the two corresponding organs were significant at the 0.1 per cent level. We can therefore conclude that there was a host difference in distribution of eggs in the visceral organs even if the animals were infected with the same strain of the parasite.

(3) *Intensity of Infection as a Factor Influencing the Distribution of Eggs of* S. Japonicum

Although it was established that distributions of the percentages of eggs of *S. japonicum* in the visceral organs of its host are influenced by the strain of the parasite and the species of the host, it seemed necessary to know whether, if the host and strain of the parasite remained the same, the type of distribution of eggs in the visceral organs would be influenced by the intensity of infection of the parasite. For this investigation, the mouse was used as the host. The intensities of infection differed in two series of mice. As mentioned above, in the first series, each mouse was infected with 2 cercariae and in the second series, with 100 cercariae. The number of mice in the 2-cercaria series were 20, 21, 30, and 30 for the C, F, J, and P strains respectively; and in the 100-cercaria series, 11, 12, 12, and 17. Although four strains of the parasite were used in this study, only the within-strain differences between the two series of different intensity of infection were compared.

Concerning the means of the percentages of eggs in different organs in the two series of different intensities of infection, the results are shown in Table 7. In general, when the intensity of infection was at the lowest level (2-cercaria infection), the means of the percentages of eggs in the liver and large intestine were significantly higher, and in the small intestine significantly lower, than in the corresponding organs of the mice with the high intensity of infection (100-cercaria infection in the present study). With the C strain, however, the difference for the eggs in the liver in the two series of infections, while not significant, was in the reverse order. The above results are, nevertheless, sufficient to indicate that the intensity of infection

TABLE 6

Means of the percentages of eggs of S. japonicum *in different visceral organs of hamsters and mice infected with 100 cercariae of either the F or the J strain*

Organs	F strain			J strain		
	Means		Significance level of difference between the means	Means		Significance level of difference between the means
	Hamsters (N = 9)	Mice (N = 12)		Hamsters (N = 12)	Mice (N = 12)	
Lungs	0.04	0.04	NS	0.09	0.001	NS
Liver	40.31	43.00	NS	14.99	11.29	<0.05
Spleen	0.02	1.79	<0.01	0.01	0.12	NS
Stomach	0.35	0.94	NS	1.24	0.11	<0.01
S. intestine	16.03	40.47	<0.001	46.24	81.27	<0.001
L. intestine	43.24	13.77	<0.001	37.42	7.20	<0.001
Total	99.99	100.01		99.99	99.99	

TABLE 7

Means of the percentages of eggs of S. japonicum *in different visceral organs of mice infected with 2 or with 100 cercariae of one of the four geographic strains*

Organs	C strain			F strain			J strain			P strain		
	Means		Significance level	Means		Significance level	Means		Significance level	Means		Significance level
	2 cercariae (N = 20)	100 cercariae (N = 11)		2 cercariae (N = 21)	100 cercariae (N = 12)		2 cercariae (N = 30)	100 cercariae (N = 12)		2 cercariae (N = 30)	100 cercariae (N = 17)	
Lungs	0.002	0.15	NS	0.01	0.04	<0.05	0.19	0.001	NS	0.05	0.02	NS
Liver	42.36	42.77	NS	54.06	43.00	<0.02	25.42	11.29	<0.001	28.89	20.55	<0.001
Spleen	0.001	0.18	NS	0.08	1.78	<0.01	0.01	0.12	NS	0.004	0.13	NS
Stomach	0.02	0.24	<0.05	0.50	0.94	NS	0.01	0.11	NS	0.01	0.29	NS
S. intestine	11.75	33.76	<0.001	16.45	40.47	<0.001	43.06	81.27	<0.001	12.67	61.91	<0.001
L. intestine	45.87	22.90	<0.001	28.89	13.77	<0.001	31.31	7.20	<0.001	58.37	17.10	<0.001
Total	100.00	100.00		99.99	100.00		100.00	99.99		99.99	100.00	

is a factor which will influence the distribution of eggs of the four strains of *S. japonicum* in the visceral organs of the host.

DISCUSSION

The fact that distributions of the percentages of eggs of *S. japonicum* in the visceral organs of its host are influenced by the strain of the parasite, the species of the host, and the intensity of infection of the parasite in the host, seems to be well established by the results of the above studies.

The existence of four distinct geographic strains of *S. japonicum* has been previously discussed by us in detail.[9] The present study gives additional support to the distinctiveness of the characteristics of the four strains.

As only two host-species, the hamster and albino mouse, were used in the present study, and as the type of distribution of eggs of *S. japonicum* in the visceral organs of the hosts has been proved to be different in host-species, our discussion will be confined to the results obtained by previous investigations of these two host-species. The reason that the distinctiveness of the type in respect to distribution of eggs of the F and J strains in the hamster was not shown in Moore and Sandground's results is most probably due to the fact that their results for the J strain were obtained from the examination of a single hamster. It is also to be noted that their animals were sacrificed 21 days after eggs were first found in the feces.

Question may arise as to how one female *japonicum* worm could deposit eggs in so many different viscera within 5 days from the first appearance of the eggs in the stools. Two possibilities may exist: (1) the mated couple might wander into one tributary of the portal vein after another, all the way from the splenic to the inferior mesenteric; and (2) the eggs might pass through the liver into the systemic circulation as their presence in the lungs suggests, and from there into the spleen, stomach, and intestinal capillaries. Judging from the fact that only a few eggs were found in the lungs, and usually there were no eggs in the other organs except the visceral ones, the second possibility seems to be remote. The first proposition may partly explain the situation. It must be added that even if the mated couple is located in a very distal branch of the mesenteric vein, eggs may be carried back to the liver by the portal blood. The portion of the vessels selected by the worms and the length of time spent in various locations will determine the types of the egg distribution as described in the present study. This leads us to consider that each strain of *S. japonicum* may have its own distinct habitat. Concerning the duration of oviposition of the worm in the animals used in the present series of experiments, our previous studies[10] have shown that in all of the strains, oviposition of the female usually started about 10 days or more before the date when the eggs could be found in the stools. The distribution of eggs in the viscera reported on

the 5th day of the first appearance in the stools should be regarded as the result of oviposition for a period of about 2 weeks.

The relationship between distribution of pathological lesions and the number of eggs in the affected organs is also interesting. In the digestive tract of mice infected with the J strain, Ito[5] found, as mentioned above, that numerous egg lesions were found at the lower duodenum and upper jejunum, while egg lesions were fewer in the other parts of the intestines. The type of distribution of eggs of the J strain in the digestive tract of this host-species as described in this paper agrees well with the type of distribution of the lesions.

It will be of great interest if the type of distribution of eggs in the digestive tract of dogs and rhesus monkeys can be studied so that Faust and Meleney's findings of pathological lesions in the dog and Vogel and Minning's findings in the rhesus monkey can be correlated. As Faust and Meleney did not specify the source of cercariae with which their dogs for the pathological studies were infected, it would be of interest to re-study the pathological lesions in dogs infected with known strains of the parasite.

The susceptibility of the rhesus monkey to infection with the four main geographic strains of *S. japonicum* is like that in man, *i.e.*, it is susceptible to the Chinese, Japanese, and Philippine strains but not susceptible to the Formosan strain.[11] If distribution of eggs and lesions are found to be different in rhesus monkeys infected with different human geographic strains of the parasite, it is possible to consider that lesions in the patients infected with *S. japonicum* in China, Japan and the Philippines may also be distinct.

SUMMARY

Distributions of eggs of the four main geographic strains of *Schistosoma japonicum* in the viscera of infected hamsters and mice were studied. The results of the study show that the percentage distributions of eggs of this parasite in the visceral organs of its host were influenced by the strain of the parasite, the species of the host, and the intensity of infection in the host. It may be stated that in general, with the C and F strains, the percentage distributions of eggs in the visceral organs of hosts were higher in the liver than in the small intestine; with the J and P strains, higher in the small intestine than in the liver except in mice infected with one male and one female cercariae of the P strain, where the mean of the percentages of eggs in the liver was higher. As to the species of host, the mean percentage of eggs in the small intestine was lower in the hamster than that in the mouse, and vice versa in the large intestine. When the intensity of infection in the host was at the lowest level, the percentages of eggs were mostly higher in the liver and large intestine, and lower in the small intestine, than in the corresponding organs of the host with the high intensity of infection.

ACKNOWLEDGMENT

We wish to express our thanks to Dr. Paul Blommers, Professor of Statistics, College of Education, State University of Iowa, for his advice in making statistical analysis of the data.

REFERENCES

1. Moore, D. V., and Sandground, J. H., 1956. The relative egg producing capacity of *Schistosoma mansoni* and *Schistosoma japonicum*. *Am. J. Trop. Med. & Hyg.*, **5**: 831–840.
2. Meleney, H. E., Sandground, J. H., Moore, D. V., Most, H., and Carney, B. H., 1953. The histopathology of experimental schistosomiasis. II. Bisexual infections with *S. mansoni*, *S. japonicum*, and *S. haematobium*. *Am. J. Trop. Med. & Hyg.*, **2**: 883–913.
3. Faust, E. C., and Meleney, H. E., 1924. Studies on schistosomiasis japonica. *Am. J. Hyg., Monog. Ser.*, **3**: 1–339.
4. Vogel, H., and Minning, W., 1953. Ueber die erworbene Resistenz von *Macacus rhesus* gegenuber *Schistosoma japonicum*. *Ztschr. Tropenmed. u. Parasitol.*, **4**: 418–505.
5. Ito, J., 1955. Studies on the host-parasite relationships of *Schistosoma japonicum* in common laboratory animals. *Jap. J. M. Sc. & Biol.*, **8**: 43–62.
6. Welch, B. L., 1947. The generalization of Student's problem when several different population variances are involved. *Biometrika*, **34**: 28–35.
7. Aspin, A. A., 1948. An examination and further development of a formula arising in the problem of comparing two mean values. *Biometrika*, **35**: 88–96.
8. Kruskal, W. H., and Wallis, W. A., 1952. Use of ranks in one-criterion variance analysis. *J. Am. Statist. A.*, **47**: 583–621.
9. Hsü, H. F., and Hsü, S. Y. Li, 1960. Characteristics of geographic strains of *Schistosoma japonicum* in the final hosts. *Proc. 6th Internat. Congr. Trop. Med. Malaria*, Lisbon, September 1958, in press.
10. Hsü, H. F., Hsü, S. Y. Li, and Tsai, C. T., 1960. Further studies on the prepatent periods of four geographic strains of *Schistosoma japonicum*. *Libre homenaje al Dr. Eduardo Caballero y Caballero.* in press.
11. Hsü, H. F., and Hsü, S. Y. Li, 1960. The infectivity of four geographic strains of *Schistosoma japonicum* in the rhesus monkey. *J. Parasitol.* in press.

STUDIES ON ECHINOCOCCOSIS: SEROLOGY OF CRUDE AND FRACTIONATED ANTIGENS PREPARED FROM *ECHINOCOCCUS GRANULOSUS* AND *ECHINOCOCCUS MULTILOCULARIS*

IRVING G. KAGAN, LOIS NORMAN AND DOROTHY S. ALLAIN

Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center, Atlanta, Georgia

In previous publications we have described evaluation studies of hemagglutination and flocculation tests for the diagnosis of hydatid disease.[1, 2] A small number of non-specific reactions was observed with both techniques with serum from patients not infected with *Echinococcus granulosus* (Eg). Sera from individuals believed to have been exposed to *Echinococcus multilocularis* (Em) were found to be reactive, but some of the antigens employed needed further study.

In this paper, experiments to measure the specificity of antigens from *E. granulosus* and *E. multilocularis* will be described. Raw hydatid fluid and crude extracts of scolices, membranes and cysts, and fractions of hydatid fluid of *E. granulosus* and cysts of *E. multilocularis* were prepared. The method of fractionation was patterned after the technic employed by Dennis.[3] Both crude antigens and the various fractions were tested with antisera prepared in rabbits, with sera from patients infected with *E. granulosus* and with sera from Eskimos believed to be infected with *E. multilocularis*. Results obtained by tannic acid hemagglutination (HA) tests and the bentonite flocculation (BF) tests will be reported.

MATERIALS AND METHODS

Tests

Hemagglutination test. The slightly modified tannic acid hemagglutination test of Boyden[4] as described in our earlier paper[1] was used. The procedure employed sheep cells in 3.8% citrate, washed 3 times in phosphate-saline buffer (pH 7.2) and adjusted to a 2.5% suspension with buffered saline. An equal volume of 1:20,000 tannic acid in buffered saline was added and the mixture incubated at 37°C for 10 minutes. After washing the cells free of excess tannic acid with buffered saline, 2 volumes of antigen were added to each volume of packed cells which were then incubated at room temperature for 15 minutes. After 2 to 3 ml of 1:250 normal rabbit serum in buffer was added to the cell-antigen mixture, it was centrifuged at 1,000 rpm for 5 minutes. The packed cells were re-suspended in 1:250 normal rabbit serum in buffer as a 2% suspension and used as antigen. Two-fold serial dilutions of sera in tubes 10 × 70 mm were made with 1:100 normal rabbit serum in buffer, starting at a dilution of 1:50. One drop of antigen-treated cells was added to 0.5 ml of diluted serum. The pattern of the erythrocytes on the bottom of the tube was read after 3 to 4 hours and again after standing at room temperature overnight. The end point of the test was a clearly discernible mat of cells across the bottom of the tube equivalent to the 4-plus reaction illustrated by Stavitsky.[5]

In the HA test all antigens were initially diluted to a point where non-specific agglutination was eliminated. Once this point was determined, several further dilutions of antigen were tested and the concentration which gave the highest titer and unequivocal negative tests was employed. Several selected sera were used to standardize each antigen, and then the entire battery of sera was tested. Positive and negative controls were included each time new cells were prepared.

Bentonite flocculation test. The BF test was described in detail in an earlier paper.[2] In summary, a suspension of standard size bentonite particles (1–2 μ in size) was mixed with antigen. After the antigen was adsorbed onto the bentonite, the excess was removed by centrifugation, the coated particles were stained with thionin and the preparation was adjusted with Tween 80 to eliminate spontaneous saline flocculation. This adjusted antigen was used by adding 1 small drop to 0.1 ml of serum on a wax-ringed slide and rotated for 15 minutes before being read. Sera diluted with saline 1:5, 1:10, and 1:20 were screened, and positive sera were titrated to an

end point. Concentrations for the fractionated antigens were determined by experiment. The criteria employed were the highest concentration of antigen which gave non-specific flocculation in saline and which could be adjusted with Tween 80 to give negative results. Positive and negative control sera were tested with each antigen prior to titrating the sera in the battery.

Antigen Preparation

1. *Crude extracts of* E. granulosus.

Hydatid fluid of *E. granulosus* was collected from cysts in pigs brought to slaughter in Memphis, Tennessee. Sixty ml of fluid aspirated from small cysts was dialyzed 24 hours in tap water and distilled water, pervaporated to 20 ml, preserved with Merthiolate (1:10,000), made isotonic with sodium chloride (0.8%) and stored frozen in 5 ml amounts. Nitrogen and carbohydrate concentrations before dialysis and pervaporation were 0.488 mg N/ml and 1.62 mg CHO/ml; afterward, 0.104 mg N/ml and 0.30 mg CHO/ml. This concentrated hydatid fluid antigen (EgHF) was used full strength in the BF tests, and was diluted 16-fold for the HA tests.

Scolices of *E. granulosus* were recovered from cysts, washed 3 times in saline by centrifugation, and lyophilized. Two hundred mg of lyophilized scolices were ground in a glass tissue grinder with 5 ml of saline, extracted 3 hours at room temperature (23°C) and 48 hours at 4°C. The suspension was centrifuged, and the supernatant used for scolices antigen (EgS) in the serological tests. Analysis of the antigen showed 0.84 mg N/ml and 2.56 mg CHO/ml. For HA tests the antigen was diluted to 0.002 mg N/ml (1:400) and for BF tests to 0.4 mg N/ml.

Membranes of *E. granulosus* were obtained from cysts that had been aspirated for hydatid fluid. When the cysts were opened, the whitish membrane found lying free in the cavity was removed, minced with scissors, washed in saline and lyophilized. An antigen was prepared by the procedure used above, but only 3 ml of antigen was recovered (0.3 mg N/ml) from 5 ml of saline, since the material was difficult to grind and very hygroscopic. This antigen (EgM) was diluted to 0.003 mg N/ml (1:100) for HA tests and concentrated to 0.4 mg N/ml for BF tests.

2. *Crude extracts of* E. multilocularis.

Hydatid fluid of *E. multilocularis* was aspirated from cysts in experimentally infected cotton rats, *Sigmodon hispidus*. Analysis of the fluid showed 5.4 mg N/ml and 0.92 mg CHO/ml. The antigen (EmHF) was stored frozen and used in a concentration of 1.30 mg N/ml for HA tests and 0.4 mg N/ml for BF tests.

Cysts from cotton rat livers were freed as much as possible from liver tissue, ground in a Waring Blendor in saline and lyophilized. Soluble extracts were prepared from 200 mg of dried material with 5 ml of saline. Two antigen preparations (EmC-CR) contained 1.24 mg N/ml and 1.98 mg N/ml, respectively. For HA tests the antigen was diluted to 0.007–0.01 mg N/ml (1:200) and for BF tests to 0.4 mg N/ml. This antigen was also used to immunize rabbits.

Cysts of *E. multilocularis* from experimentally infected gerbils, *Meriones unguiculatus*, were collected from animals infected by intraperitoneal injection of viable cyst material. After 5 months of infection, cysts free in the peritoneal cavity were collected and a soluble antigen (EmC-G) prepared as described above (0.94 mg N/ml). For HA tests this antigen was diluted to 0.0045 mg N/ml (1:200) and for BF tests to 0.4 mg N/ml.

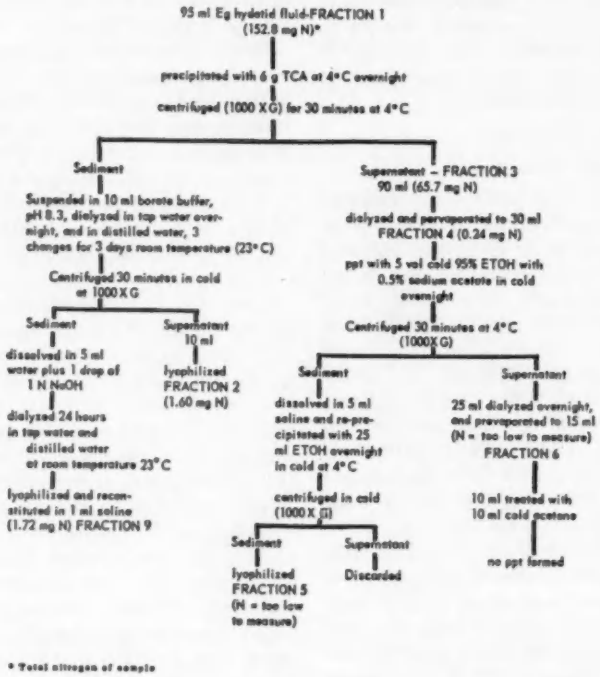
Scolices of *E. multiocularis*, separated from cyst tissues, were obtained from small cysts in cotton rats. They were disrupted by homogenization in a Waring Blendor for 30 seconds, concentrated by light centrifugation, washed 3 times, and lyophilized. Three lots of soluble antigen (EmS) prepared by using 200 mg of lyophilized scolices ground in 5 ml of saline yielded antigens with concentrations of 0.55 mg N/ml, 0.52 mg N/ml and 0.68 mg N/ml. This antigen was diluted 200-fold for HA tests and to 0.4 mg N/ml for BF tests.

3. *Preparation of echinococcus fractions.*

E. granulosus. Hydatid fluid, obtained from cysts in livers of pigs, was stored frozen for several months and was fractionated according to the methods outlined in Table 1. Total nitrogen was determined for the 7 fractions obtained. For HA tests the following concentrations were used: Fraction 1, 0.271 mg N/ml; Fraction 2, 0.08 mg N/ml; Fraction 4, 0.04 mg N/ml; Fraction 5 and 6 were combined, insufficient amount for chemical analysis; Fraction 9, 0.172 mg N/ml. For BF tests the following concentrations were used: Fraction 1, 1.91 mg N/ml; Fraction 2, 0.16 mg N/ml; Fraction 4, 0.08 mg N/ml; Fractions 5 and 6, combined and

TABLE 1

FRACTIONATION OF *ECHINOCOCCUS GRANULOSUS* HYDATID FLUID


95 ml Eg hydatid fluid-FRACTION 1
(152.8 mg N)*
precipitated with 6 g TCA at 4°C overnight
centrifuged (1000 X G) for 30 minutes at 4°C
Sediment
Suspended in 10 ml borate buffer, pH 8.3, dialyzed in tap water overnight, and in distilled water, 3 changes for 3 days room temperature (23°C)
Centrifuged 30 minutes in cold at 1000 X G
Sediment
dissolved in 5 ml water plus 1 drop of 1 N NaOH
dialyzed 24 hours in tap water and distilled water at room temperature 23°C
lyophilized and reconstituted in 1 ml saline (1.72 mg N) FRACTION 9
Supernatant 10 ml
lyophilized FRACTION 2 (1.60 mg N)
Supernatant – FRACTION 3
90 ml (65.7 mg N)
dialyzed and pervaporated to 30 ml FRACTION 4 (0.24 mg N)
ppt with 5 vol cold 95% ETOH with 0.5% sodium acetate in cold overnight
Centrifuged 30 minutes at 4°C (1000 X G)
Sediment
dissolved in 5 ml saline and re-precipitated with 25 ml ETOH overnight in cold at 4°C
centrifuged in cold (1000 X G)
Sediment
lyophilized FRACTION 5 (N = too low to measure)
Supernatant
Discarded
Supernatant
25 ml dialyzed overnight, and pervaporated to 15 ml (N = too low to measure) FRACTION 6
10 ml treated with 10 ml cold acetone
no ppt formed

* Total nitrogen of sample

used full strength; Fraction 9, 0.172 mg N/ml. All fractions used in the BF test were brought to pH 7 with borate buffer (pH 8.3) or 0.1 N HCl prior to use.

E. multilocularis. Cysts from experimentally infected gerbils were fractionated according to the methods outlined in Table 2. A total of 11 fractions was obtained. In the HA tests, the following concentrations were employed: Fraction A, 0.006 mg N/ml; Fraction D, 0.001 mg N/ml; Fraction I, 0.0075 mg N/ml; Fraction J, 0.008 mg N/ml; Fraction K, 0.011 mg N/ml. In the BF tests, the following concentrations were used: Fraction A, 0.35 mg N/ml; Fraction D, 0.3 mg N/ml; Fraction E, 0.3 mg N/ml; Fraction F, 0.144 mg N/ml; Fraction G, 0.236 mg N/ml; Fraction I, 0.22 mg N/ml; Fraction J, 0.23 mg N/ml; Fraction K, 0.28 mg N/ml.

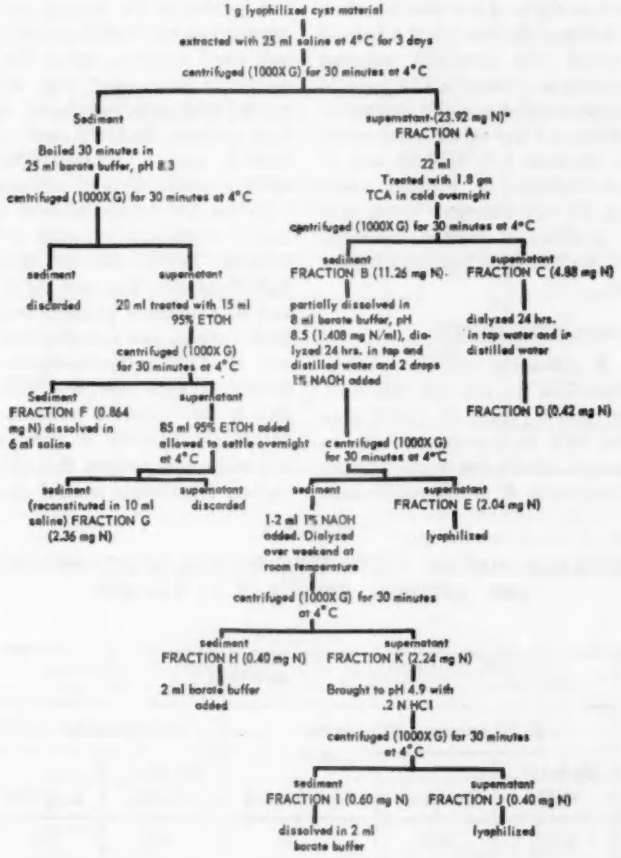
Nitrogen values for all fractions were measured by means of a micro-Kjeldahl method[6] and total polysaccharides with anthrone reagent.[7] All antigens were preserved with Merthiolate (1:10,000).

Antisera Preparation

Saline suspensions of lyophilized antigens and liquid hydatid fluid were mixed with an equal amount of Freund's adjuvant (85 ml Bayol F, 15 ml Arlacel A and 10 mg lyophilized BCG tubercle organisms) for rabbit immunizations. Rabbits were inoculated subcutaneously once each week for 4 weeks with 1 ml of antigen-adjuvant mixture in several sites on the back and shoulder. After a rest period of 15 to 21 days, each rabbit was bled to death by severing the femoral artery. Serum removed from the clot was Merthiolated (1:10,000) and stored at −20°C until used. At least two rabbits were used for each echinococcus antigen. A third rabbit was immunized with a combination of Eg hydatid fluid in Freund's adjuvant and 2 ml of liquid hydatid fluid administered intravenously. Only

TABLE 2

FRACTIONATION OF *ECHINOCOCCUS MULTILOCULARIS* CYST ANTIGEN


1 g lyophilized cyst material
extracted with 25 ml saline at 4°C for 3 days
centrifuged (1000X G) for 30 minutes at 4°C
Sediment
Boiled 30 minutes in 25 ml borate buffer, pH 8.3
centrifuged (1000X G) for 30 minutes at 4°C
sediment
discarded
supernatant
20 ml treated with 15 ml 95% ETOH
centrifuged (1000X G) for 30 minutes at 4°C
Sediment
FRACTION F (0.864 mg N) dissolved in 6 ml saline
supernatant
85 ml 95% ETOH added allowed to settle overnight at 4°C
sediment (reconstituted in 10 ml saline) FRACTION G (2.36 mg N)
supernatant discarded
supernatant-(23.92 mg N)*
FRACTION A
22 ml
Treated with 1.8 gm TCA in cold overnight
centrifuged (1000X G) for 30 minutes at 4°C
sediment
FRACTION B (11.26 mg N)-
partially dissolved in 8 ml borate buffer, pH 8.5 (1.408 mg N/ml), dialyzed 24 hrs. in tap and distilled water and 2 drops 1% NAOH added
centrifuged (1000X G) for 30 minutes at 4°C
sediment
1-2 ml 1% NAOH added. Dialyzed over weekend at room temperature
supernatant
FRACTION E (2.04 mg N)
lyophilized
centrifuged (1000X G) for 30 minutes at 4°C
sediment
FRACTION H (0.40 mg N)
2 ml borate buffer added
supernatant
FRACTION K (2.24 mg N)
Brought to pH 4.9 with .2 N HCl
centrifuged (1000X G) for 30 minutes at 4°C
sediment
FRACTION I (0.60 mg N)
dissolved in 2 ml borate buffer
supernatant
FRACTION J (0.40 mg N)
lyophilized
supernatant
FRACTION C (4.88 mg N)
dialyzed 24 hrs. in tap water and in distilled water
FRACTION D (0.42 mg N)

* Total nitrogen of sample

one rabbit was inoculated with cotton rat liver. The following total amounts of nitrogen were administered: EgHF, 1.66 mg, 2.35 mg and 1.80 mg; EgS, 0.28 mg; EgM, 0.86 mg; EmHF, 13.5 mg; EmC-CR, 1.26 mg; EmS, 0.456 mg and 3.22 mg; cotton rat liver, 1.10 mg. In this study the antiserum with the highest titer by flocculation when tested with its homologous antigen was used for titration with the other antigens.

Sources of Human Sera

Clinical histories of patients with E. granulosus. Eleven human sera were available for study. Specimens E3, E679, E1990, E4, E73, E257, D, and E1024 were obtained from patients with diagnoses of hydatid disease confirmed by surgery. Patient M had a large hydatid cyst in the liver, but was not surgically treated. Patient E338 had a positive Casoni intradermal reaction and radiological evidence of calcified cysts, and E364 was an 8-year old girl with a lung hydatid cyst diagnosed radiologically.

Clinical histories of patients with suspected infections of E. multilocularis. Thirteen sera were obtained in 1958 from Eskimos living in two

communities on St. Lawrence Island in the Bering Sea, where *E. multilocularis* is endemic and *E. granulosus* does not occur. The epidemiology of *E. multilocularis* in this area has been described by Rausch and Schiller.[8] Patient 1 had a hepatic hydatid cyst which was surgically removed several years previously. Patient 2 had hydatid cysts surgically removed from the peritoneal cavity in 1953. Patient 3 had enlargement of the spleen and liver. Patients 4, 5, 6, 7, 14, and 18 had radiological evidence of hydatid cysts. Patients 10 and 12 had enlarged livers, and patient 9 gave evidence of hepatic disorders. Patient 15 had *E. multilocularis* hydatid infection proven by biopsy.

EXPERIMENTAL RESULTS

All the crude *E. granulosus* and *E. multilocularis* antigens reacted in the HA test with antisera prepared in rabbits (Table 3). EgHF gave the highest titers with its homologous antisera and with EgS antisera, and a low degree of cross reactivity was noted with *E. multilocularis* antisera. EgS reacted equally well with the homologous scolices and heterologous Em cyst antisera. EgM was not as sensitive as the other Eg antigens but reacted at the highest titer with its homologous antisera. EmHF reacted best with EmHF and EmC antisera, and a low degree of cross reactivity was noted with Eg antisera. EmS reacted best with homologous antisera and with EgS antisera. EmC-CR was not as reactive as EmC-G antigen. EmC-G showed little to no specificity with the antisera tested.

In the BF tests (Table 4) both EgHF and EmHF were more reactive with their specific antisera. EmHF did not react with EgS or EgHF antisera. EgS and EgM reacted with all anti-*E. granulosus* antisera and with EmS and EmC antisera, but not with EmHF. EmS reacted with all of the anti-echinococcus sera at approximately the same titer. EmC-CR reacted with all anti-*E. multilocularis* antisera, but only with anti-EgS antiserum; it seems noteworthy that this is the only antigen that did not produce the highest titers when reacted against homologous

TABLE 3

HEMAGGLUTINATION TESTS WITH ECHINOCOCCUS ANTIGENS AND ANTISERA PREPARED IN RABBITS

Antigens	Antisera					
	Echinococcus granulosus			*Echinococcus multilocularis*		
	Hydatid fluid	Scolices	Membranes	Hydatid fluid	Scolices	Cysts
Eg HF	3200	3200	0	200	100	100
Eg S	800	3200	–	0	0	6400
Eg M	0	200	800	0	50	0
Em HF	400	200	0	25600+	200	12800
Em S	100	3200	0	0	25600+	400
Em C-CR	200	200	0	6400	3200+	3200
Em C-G	3200	6400	1600	800	12800	800

Eg – *Echinococcus granulosus*
Em – *Echinococcus multilocularis*
HF – Hydatid fluid
S – scolices
M – membrane
C – cysts
Note – Titers expressed as reciprocals

TABLE 4

FLOCCULATION TESTS WITH ECHINOCOCCUS ANTIGENS AND ANTISERA PREPARED IN RABBITS

Antigens	Antisera					
	Echinococcus granulosus			*Echinococcus multilocularis*		
	Hydatid fluid	Scolices	Membranes	Hydatid fluid	Scolices	Cysts
Eg HF	40	20	10	0	10	0
Eg S	10	20	10	0	10	5
Eg M	10	10	10	0	5	5
Em HF	0	0	0	160	160	0
Em S	20	40	10	10	20	20
Em C-CR	0	5	0	320	5	40
Em C-G	20	20	0	20	0	10

Eg — *Echinococcus granulosus*
Em — *Echinococcus multilocularis*
HF — hydatid fluid
S — scolices
M — membrane
C — cysts
Note — Titers expressed as reciprocals

TABLE 5

HEMAGGLUTINATION TESTS WITH ECHINOCOCCUS ANTIGENS AND SERA FROM PROVEN CASES OF *ECHINOCOCCUS GRANULOSUS*

	Serum Number										
Antigens	E3	E4	E73	E257	E1990	E679	M	D	E1024	E338	E364
Eg HF	12800	25600+	25600+	800	3200	3200	400	25600+	25600+	6400	25600+
Eg S	6400	3200	3200	50	3200+	–	200	3200+	–	100	3200+
Eg M	100	0	0	0	800	–	0	3200+	–	50	0
Em HF	200	0	0	0	1600	–	50	3200+	–	800	0
Em S	200	400	0	0	–	–	0	3200+	–	200	1600
Em C-CR	100	200	0	0	–	–	0	200	–	200	0
Em C-G	200	1600	0	50	400	1600	200	3200	3200	0	800

Eg — *Echinococcus granulosus*
Em — *Echinococcus multilocularis*
HF — hydatid fluid
S — scolices
M — membranes
C — cysts
Note — Titers expressed as reciprocals

antiserum. EmC-G produced essentially the same titers with EmHF antisera but had no reaction with EmS; cross reactivity was noted with EgHF and with EgS antisera but not with EgC antisera.

Tests with antisera from patients infected with *E. granulosus* indicated that EgHF was the most reactive antigen in both HA (Table 5) and BF (Table 6) tests. EgS, EmC-CR, EmC-G were reactive in the BF test with rabbit antisera (Table 4) but were not as sensitive when tested in the BF test with human sera (Table 6).

Hemagglutination tests with sera from individuals suspected of harboring hydatid cysts of *E. multilocularis* showed that EgHF was more sensitive than EmHF in detecting an active infection in patient 15 (Table 7). The relatively high titers obtained with this serum indicated active infection which was later verified by biopsy. Of the 4 Em antigens utilized in the HA test, EmC-G appeared to be the most sensitive. In the BF test, EmHF was the only antigen which showed a titer of 1:5 against antisera 1 and 2 (proven cases). EgHF showed a titer of 1:5 with serum 15 and with serum 10. All the antigens were active with serum 10. None of the crude antigens employed with these sera was adequate in the BF test.

Not all of the echinococcus fractions were reactive in the HA and BF tests. Only fractions 1, 2 and 9 from hydatid fluid were active in the HA tests. Fraction 3 could not be used because the TCA lysed red cells and fractions 4, 5, and 6 did not give positive reactions with any control sera. In the BF test, 4 fractions (1, 2, 5-6 and 9) were active. Fractions 3 and 4 were not adsorbed by the bentonite. Fractions 5 and 6 were combined for use; however, even when combined, there was not sufficient antigenic material for use except in testing 7 rabbit and 2 human sera. The fine floccules produced in reactions with Fraction 2 were difficult to evaluate; consequently, this was considered a poor flocculating antigen.

Although all of the fractions prepared from cysts of *E. multilocularis* were tested, only A, D, K and I were suitable for HA tests, and only A, E, K, I and J were suitable for BF tests. Fraction B was not tested serologically, but nitrogen determination was made. The TCA in Fraction C lysed red blood cells and probably prevented adsorption of antigen onto bentonite.

Serological results with antigen fractions and

TABLE 6

FLOCCULATION TESTS WITH ECHINOCOCCUS ANTIGENS AND SERA FROM PROVEN CASES OF *ECHINOCOCCUS GRANULOSUS*

	Serum Number										
Antigens	E3	E4	E73	E257	E1990	E679	M	D	E1024	E338	E364
Eg HF	320	1280	160	0	20	640	10	2560	1280	10	640
Eg S	0	10	10	0	0	0	0	10	0	0	40
Eg M	0	5	0	0	0	0	0	0	0	0	10
Em HF	0	0	0	0	0	0	0	10	0	0	10
Em S	0	10	0	0	0	0	5	20	0	0	40
Em C-CR	0	0	0	0	0	0	0	0	0	0	0
Em C-G	0	20	0	0	0	0	0	0	0	0	40

Eg — *Echinococcus granulosus*
Em — *Echinococcus multilocularis*
HF — hydatid fluid
S — scolices
M — membranes
C — cysts
Note — Titers expressed as reciprocals

TABLE 7

HEMAGGLUTINATION TITERS WITH ECHINOCOCCUS ANTIGENS AND SERA FROM ESKIMOS SUSPECTED OF HARBORING *ECHINOCOCCUS MULTILOCULARIS*

	Serum Number												
Antigens	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**10**	**12**	**14**	**15**	**18**
Eg HF	0	0	0	0	100	0	0	0	100	100	0	3200	0
Eg S	0	0	–	0	0	0	0	0	–	0	0	1600	0
Eg M	0	0	–	0	0	0	0	0	–	0	0	0	0
Em HF	0	–	–	50	0	0	50	0	0	50	100	400	0
Em S	0	–	–	0	–	0	0	–	–	0	0	0	0
Em C-CR	0	0	0	0	0	0	0	50	50	0	50	400	0
Em C-G	1600	0	0	0	200	0	0	100	0	0	0	3200	0

Eg – *Echinococcus granulosus*
Em – *Echinococcus multilocularis*
HF – hydatid fluid
S – scolices
M – membranes
C – cysts
Note – Titers expressed as reciprocals

TABLE 8

HEMAGGLUTINATION TESTS WITH FRACTIONATED *ECHINOCOCCUS* ANTIGENS AND ANTISERA PREPARED IN RABBITS

Anti-serum / Antigens	*Echinococcus granulosus*			*Echinococcus multilocularis*			Cotton Rat liver
	Hydatid fluid	Scolices	Membranes	Hydatid fluid	Scolices	Cysts	
Eg hydatid fluid – 1	25,600+	1600	200	1600	400	200	0
" – 2	3200	800	100	6400	400	50	0
" – 9	25,600+	6400	1600	800	400	100	50
Em cysts – A	6400	6400	200	3200	3200	800	200
" – D	200	0	–	0	0	0	0
" – K	25,600+	12,800	800	25,600+	0	400	3200
" – I	25,600+	6400	1600	25,600+	1600	800	12,800

1. – Hydatid fluid
2. – Supernatant after TCA ppt.
9. – Insoluble fraction after TCA ppt.
A. – Saline extract
D. – Supernatant after TCA ppt.
K. – TCA ppt. + NaOH
I. – Acid insoluble fraction of K.
Note – Titers expressed as reciprocals

with crude antigens (Antigens 1 and A) against homologous and heterologous rabbit antisera are shown in Tables 8 and 9. EgHF, although not dialyzed and concentrated as in the previous experiments, was a very sensitive antigen. It did not show cross reactivity with antisera to cotton rat liver. Other fractions with a sensitivity equal to or better than that of EgHF showed cross reactivity with cotton rat liver antisera as follows: EgHF-9, 1:50; Em cysts—A, 1:200; Em cysts—K, 1:3200; Em cysts—I, 1:12800. In HA tests the fractions derived from the supernatant of the TCA precipitation of hydatid fluid (4, 5 and 6) were not reactive; however, in the BF tests (Table 9) fractions 5 and 6 (which were combined) were reactive. In both the HA and BF tests Fraction 2 of hydatid fluid was not as active as Fraction 9.

Fraction K from cysts of *E. multilocularis* was prepared in the same manner as Fraction 9 from hydatid fluid of *E. granulosus*. Fraction I, a further purification of K, cross-reacted at a higher titer with cotton rat liver antiserum in the HA, but not in the BF tests. Fraction J was active in the BF, but not in the HA tests. In both tests, extracts of the saline insoluble material (Fractions F and G) were inactive.

Results of tests with human sera from individuals with *E. granulosus* infection indicate that in some cases Fraction 9 was more sensitive than K or I in both the HA and BF tests. In the HA test (Table 10) Fraction 2 proved to be as reactive as EgHF with human sera. For cyst antigens, Fraction K had about the same sensitivity as the crude antigen (Fraction A). Fraction I was the most sensitive of all the Em fractions tested. In the BF test (Table 11), Fraction K was slightly more sensitive than I, and both were better than A. Fraction D was active in HA tests but did not react in BF tests. Fractions E and J were active in the BF test with rabbit antisera but reacted with only E364 in the human sera. Em antigens from cysts were inferior to those from Eg hydatid fluid in tests with serum from infected humans.

Tests with sera from individuals suspected of having *E. multilocularis* infections indicated that EgHF was positive with 8 of 13 sera in the HA

TABLE 9

FLOCCULATION TESTS WITH FRACTIONATED *ECHINOCOCCUS* ANTIGENS AND ANTISERA PREPARED IN RABBITS

Antigens \ Antiserum	*Echinococcus granulosus*			*Echinococcus multilocularis*			Cotton Rat liver
	Hydatid fluid	Scolices	Membranes	Hydatid fluid	Scolices	Cysts	
Eg hydatid fluid – 1	80	0	0	0	0	0	0
" – 2	20	10	20	5	0	0	0
" – 5-6	10	10	0	0	0	10	5
" – 9	320	10	20	20	0	10	0
Em cysts – A	10	20	0	5	5	5	0
" – E	20	20	0	0	10	5	0
" – K	80	40	20	80	10	40	10
" – I	320	20	5	160	5	40	0
" – J	20	10	0	0	0	10	0

1. – Hydatid fluid
2. – Supernatant after TCA ppt.,
5-6. – Supernatant of TCA ppt. precipitated with ETOH
9. – Insoluble fraction after TCA ppt.
A. – Saline extract
E. – Supernatant of fraction D
K. – TCA ppt. + NaOH
I. – Acid insoluble fraction of K.
J. – Acid soluble fraction of K.

Note – Titers expressed as reciprocals

TABLE 10

HEMAGGLUTINATION TESTS WITH ECHINOCOCCUS ANTIGENS AND SERA FROM PROVEN CASES OF *ECHINOCOCCUS GRANULOSUS* IN MAN

Antigens \ Anti-serum	E3	E4	E73	E1990	E257	E679	M	D	E1024	E338	E364
Eg hydatid fluid – 1	12800	25600+	25600+	25600+	800	25600+	200	25600+	25600+	3200	25600+
" – 2	3200	25600+	25600+	25600+	800	25600+	800	25600+	25600+	1600	25600+
" – 9	6400	12800	3200	6400	400	25600+	200	25600±	25600±	3200	25600+
Em cysts – A	200	1600	0	400	50	1600	200	3200	3200	0	800
" – D	–	1600	0	400	400	100	–	400	–	0	–
" – K	400	6400	400	800	0	1600	400	6400	3200	1600	400
" – I	3200	12800	1600	800	100	3200	200	3200	1600	100	1600

1. – Hydatid fluid
3. – Supernatant after TCA ppt.
9. – Insoluble fraction after TCA ppt.
A. – Saline extract
D– Supernatant after TCA ppt.
K. – TCA ppt. + NaOH
I. – Acid insoluble fraction of K.
Note – Titers expressed as reciprocals

TABLE 11

FLOCCULATION TESTS WITH ECHINOCOCCUS ANTIGENS AND SERA FROM PROVEN CASES OF *ECHINOCOCCUS GRANULOSUS* IN MAN

Antigens \ Anti-serum	E3	E4	E73	E1990	E257	E679	M	D	E1024	E338	E364
Eg hydatid fluid – 1	320	1280	320	40	0	640	10	1280	2560	10	2560
" – 2	5	40	0	0	0	20	0	10	10	0	10
" – 9	160	2560	640	160	0	640	160	1280	640	5	1280
" – 5-6	0	10	–	–	–	–	–	–	–	–	–
Em cysts – A	0	5	0	0	0	0	0	0	0	0	40
" – E	0	0	0	0	0	0	0	0	0	0	10
" – K	0	20	0	0	0	0	5	0	5	0	80
" – I	0	5	0	0	0	0	5	0	0	0	10
" – J	0	0	0	0	0	0	0	0	0	0	40

1. – Hydatid fluid
2. – Supernatant after TCA ppt.
9. – Insoluble fraction after TCA ppt.
5-6. – Supernatant of TCA ppt., precipitated with ETOH
A. – Saline extract
E. – Supernatant – TCA ppt.
K. – TCA ppt. + NaOH
I. – Acid insoluble fraction of K.
J. – Acid – soluble fraction of antigen K.
Note – Titers expressed as reciprocals

TABLE 12

HEMAGGLUTINATION TESTS WITH FRACTIONS OF *E. GRANULOSUS* HYDATID FLUID AND *E. MULTILOCULARIS* CYST ANTIGENS WITH SERA FROM ESKIMOS SUSPECTED OF HARBORING *E. MULTILOCULARIS*

Anti-serum / Antigens	Serum Number												
	#1	#2	#3	#4	#5	#6	#7	#9	#10	#12	#14	#15	#18
Eg hydatid fluid – 1	100	0	50	50	0	0	0	50	200	100	0	6400	100
" – 2	800	0	50	0	400	0	0	0	0	0	50	12800	0
" – 9	800	50	0	0	200	0	0	0	0	0	0	3200	0
Em cysts – A	0	0	0	0	0	0	0	0	0	0	0	3200	0
" – D	–	–	–	0	–	0	–	0	–	–	–	0	–
" – K	1600	1600	200	200	200	0	0	0	0	0	0	200	0
" – I	100	50	50	50	100	50	100	0	200	100	100	1600	50

1. – Hydatid fluid
2. – Supernatant after TCA ppt.
9. – Insoluble fraction after TCA ppt.
A. – Saline extract
D – Supernatant after TCA ppt.
K. – TCA ppt. + NaOH
I. – Acid insoluble fraction of K.
Note – Titers expressed as reciprocals

test, whereas the fractions reacted only with 6 sera (Table 12). With 5 of the sera (1, 2, 5, 14, 15) the fractions of hydatid fluid did, however, show higher titers. In the HA test fractions from multilocular cysts were also more reactive than the crude antigens. Sera 1 and 2, from old proven cases, gave relatively high HA titers with Fraction K, but serum 15 (active case) did not. Serum 15 showed higher titers with hydatid fluid, fractions of hydatid fluid and with Em Fractions A and I. In the BF test crude hydatid fluid antigen showed a titer of 1:5 with sera 2 and 14, and a titer of 1:10 with serum 15. Except for a few isolated instances, almost all the antigen fractions of hydatid fluid and Em cyst-antigen were inactive.

During the fractionation of both the hydatid fluid and cyst antigens a large loss of nitrogen occurred during dialysis. Fraction 3 (Table 1) contained 65.7 mg N originally and only 0.24 mg N after dialysis (Fraction 4). With cyst material Fraction C contained 4.88 mg N, which after dialysis was reduced to 0.42 mg N (Table 2). There was an unexplained loss of N after precipitation with TCA and with 0.2 N HCl.

DISCUSSION

When hydatid fluid from the same host species was prepared in an identical manner, the reproducibility of the hemagglutination and bentonite flocculation tests as noted in previous publications[1, 2] was found to be very high. Tests with other crude echinococcus antigens were also reproducible with individual batches of antigen, since titers of selected sera did not vary more than 1 to 2 dilutions when retested many times over an extended period of several weeks. Differences were found in the sensitivity of antigens prepared by slightly different techniques. Thus, dialyzed concentrated EgHF gave different titers (Tables 3–7) than raw untreated EgHF (Tables 8–12). Comparisons of the titers obtained with two batches of EmC-G antigen (used in tests in Tables 3–7 and 8–12) showed identical titers in 34 sera, 1-dilution difference in 4 sera, 2-dilution difference in 5 sera, and 3-to 5-dilution difference in only 3 sera for the HA and BF tests.

In the serological tests there was a lack of specificity with the crude Eg and Em antigens. Since the antigens employed were raw hydatid fluid and complex saline extracts of lyophilized

materials, these results were not surprising. EmC-CR was probably slightly contaminated with host liver as the cysts could not be completely freed of host tissue during preparation. The cyst antigen from gerbils (EmC-G) did not, it is believed, contain liver tissue since the cysts were recovered from the peritoneal cavity.

Since antisera prepared against the various crude antigens undoubtedly varied in antibody concentration, the relative degrees of specificity of the antigens obtained in the tests cannot be compared. Moreover, since the concentration of the various antigens which can be employed in the HA test varies considerably, the absolute titers obtained with different antigens should, and indeed do, vary. The titers obtained are not intended as a comparative estimation of the inherent antigenicity of the various fractions, but rather a comparison of these antigens as they may be used as laboratory diagnostic reagents with the two tests employed. For diagnostic purposes, hydatid fluid of *E. granulosus* appears to be the antigen of choice at the present time.

There appears to be little correlation between serological reactivity of an antigen and its total nitrogen. Nitrogen values were determined only for standardization and as a basis for duplicating test materials. In the BF test 0.4 mg N/ml appears to be a concentration which gives maximum sensitivity with the antigens employed.

The poor titers with sera from humans believed to be infected with *E. multilocularis* when tested with crude and fractionated antigens may perhaps be explained. The sera were obtained from individuals with spontaneously cured infections or with calcified cysts, or from individuals that might not have been infected. With serum 15 from an individual with a verified active infection, HA titers with the crude antigens were high (Tables 7 and 12). For diagnostic purposes all of the remaining sera tested with crude antigens, except serum 1 which was tested with EmC-G in the HA test, were negative since titers below 1:200 in the HA tests are not considered specific for hydatid disease.[1] When these sera were tested in the HA test with purified antigens (Table 12) sera 1, 2, and 15 (all proven cases) were positive. These results may indicate that the HA or BF tests utilizing crude antigens will not detect chronic infections with *E. multilocularis*.

Fraction I of Em cyst material was the antigen which most closely resembles Dennis' antigen.[3] Dennis precipitated hydatid fluid with 3% TCA, dissolved the precipitate by adding 10% sodium hydroxide drop by drop to a water suspension of the precipitate and reprecipitated with 1 N glacial acetic acid. In the present study 0.2 N HCl was used for final precipitation instead of glacial acetic acid and fractions were dialyzed. Fraction 9 of the hydatid fluid series closely approaches the Dennis type antigen, but it was not reprecipitated. Both fractions Em-I and EgHF-9 were more reactive than the unfractionated materials, but in HA they were less specific in that they cross-reacted with cotton rat liver antiserum. As will be shown in another paper, the presence of host protein in echinococcus antigens may be responsible for non-specific serological reactions with sera from patients ill with non-helminthic diseases.[9] The Dennis type antigen increased the sensitivity of the serological test, but at the same time it might have concentrated non-specific host factors.

There appears to be a marked difference in the sensitivity of the BF test with rabbit antisera and with human diagnostic sera since some antigen fractions reacted well with the former and very poorly with the latter. In the HA test this phenomenon was not as marked. The difference in reactivity in the BF and HA tests with the two types of sera may be due to a quantitative difference in the antibody threshold of reaction, or perhaps to the fact that in human sera the BF test may be measuring different antibodies than the HA test. It should be noted that serum E257, which gave no reaction by BF and only low titers by HA, is from a patient with a history of having had a cyst removed several years ago. Until more data have been obtained, it will be impossible to determine whether this type of serological reaction represents residual antibody to a past infection.

Since whole hydatid fluid antigen was just as reactive as the antigenic fractions in tests with human sera from patients with echinococcosis, it appears that the fractions tested offer little advantage for diagnosis. For the diagnosis of *E. multilocularis* infections, antigen fractions were more sensitive than the whole antigen, and their utilization appears to be warranted. Towards this goal, improvements will have to be made to increase the specificity of antigenic fractions prepared from the multilocular cyst material.

Fractionation of echinococcus antigens with

TCA may not be the best procedure since many of the fractions did not have the specificity and reactivity desired. The basic nature of the chemical properties of echinococcus antigens is not well understood. Hiles[10] used alcoholic extracts of scolices from hydatid cysts in his serological work and found them more potent than the hydatid fluid. He believed the antigenic factor to be alcohol-soluble rather than saline- or acetone-soluble and to be mainly lipoidal in nature. The active elements of Em antigens in our tests were saline soluble since the insoluble fraction could not be used. Analysis of some of the Eg and Em antigens with the Beckman DU spectrophotometer in the ultraviolet band revealed no tyrosine or tryptophane peaks. These findings suggest that perhaps protein may not be a major reactive component in our antigens. Analysis of schistosome antigens indicates that they are essentially lipo-protein in nature.[11] The fact that it was possible to measure polysaccharide in the echinococcus antigens suggests that methods of extracting lipo-protein-polysaccharide complexes may prove effective in the isolation of active serological antigens.

A loss of nitrogen was noted during the fractionation of the antigens after both dialysis and precipitation with TCA or HCl. In the hydatid fluid fractionation the difference in nitrogen between Fraction 3 and Fraction 4 indicated that most of the protein nitrogen was precipitated by the TCA. Until further studies have been made, Kjeldahl nitrogen should be used as a quantitative estimation of antigens; but future work perhaps will reveal a better method.

SUMMARY

Hemagglutination (HA) and bentonite flocculation (BF) tests with 7 crude *Echinococcus granulosus* and *Echinococcus multilocularis* antigens were performed. Antisera prepared in rabbits against these antigens, antisera from patients with proven infections of *E. granulosus* and serum from patients believed to be infected with *E. multilocularis* were titrated. In both the HA and BF tests absolute specificity was not observed with hydatid fluid, scolices, cyst and membrane antigens of the two cestode species. *E. granulosus* hydatid fluid proved to be the antigen of choice in both the HA and BF tests of sera from humans with *E. granulosus* hydatid infections. Diagnostic tests with *E. multilocularis* antisera were not as satisfactory, suggesting that tests with crude antigens will not detect human chronic infections with this species of parasite.

By precipitation with TCA, 7 fractions were obtained from *E. granulosus* hydatid fluid, and 11 from *E. multilocularis* cysts. A number of these fractions were non-reactive in both the HA and BF tests. Fractionated antigens were more sensitive than whole antigens when used with antisera prepared in rabbits. For diagnostic serology, the purified antigens were in some instances more active in the HA test but less reactive in the BF test. For the diagnosis of *E. granulosus* hydatid infection, the whole hydatid fluid of porcine origin was as reactive as the antigen fractions. For the diagnosis of *E. multilocularis* hydatid infections, the fractionated antigens prepared from whole cysts were superior.

ACKNOWLEDGMENTS

We wish to express our appreciation to Dr. T. C. Utley, Veterinarian in charge, and Dr. P. H. Mommsen of the Meat Inspection Division of the U. S. Department of Agriculture for collecting and shipping hydatid fluid and cysts in infected livers; and to Dr. Robert Rausch, Arctic Health Center, Public Health Service, Anchorage, Alaska.

REFERENCES

1. Kagan, I. G., Allain, D. S., and Norman, L., 1959. An evaluation of the hemagglutination and flocculation tests in the diagnosis of echinococcus disease. *Am. J. Trop. Med. & Hyg.*, **8:** 51–55.
2. Norman, L., Sadun, E. H., and Allain, D. S., 1959. A bentonite flocculation test for the diagnosis of hydatid disease in man and animals. *Am. J. Trop Med. & Hyg.*, **8:** 46–50.
3. Dennis, E. W., 1937. A stable concentrated purified antigen for the immunological study of hydatid disease. *J. Parasitol.*, **23:** 62–67.
4. Boyden, S. V., 1951. The adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. *J. Exper. Med.*, **93:** 107–120.
5. Stavitsky, A. B., 1953. Micromethods for the study of proteins and antibodies. 1. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. *J. Immunol.* **72:** 360–367.
6. Kabat, E. A., and Mayer, M. M., 1948. *Experimental Immunochemistry.* Charles C Thomas, Springfield.
7. Dische, Z., 1955. New color reactions for

determination of sugars in polysaccharides. in *Methods of Biochemical Analysis*, ed. by David Gluck, vol. 2, pp. 313–358. Interscience Pub., Inc., New York.

8. Rausch, R., and Schiller, E. L., 1956. Studies on the helminth fauna of Alaska. XXV. The ecology and public health significance of *Echinococcus sibiricensis* Rausch & Schiller, 1954 on St. Lawrence Island. *Parasitology*, **46**: 395–419.
9. Kagan, I. G., Norman, L., and Allain, D. S. Studies on echinococcosis: Non-specific reactions of hydatid-fluid antigen with serum of patients ill with diseases other than echinococcosis. *J. Immunol.* In press.
10. Hiles, I., 1926. Serological studies on hydatid. *J. Helminthol.* **4**: 143–178.
11. Eliakim, M., and Davies, A. M., 1954. The complement-fixation test in bilharziasis. I. The value of different extracts of *Schistosoma mansoni* and *Fasciola hepatica* worms as antigens. *Parasitology*, **44**: 403–413.

PREVALENCE OF INTESTINAL PROTOZOA IN ADULTS AT FOUR MILITARY BASES IN CALIFORNIA*

R. L. BROWN† AND M. J. GARBER‡

This survey was designed to study the prevalence of the intestinal protozoa in a large sample of an adult population in the United States. Previous studies have surveyed more restricted groups. Confidence in results reported herein was improved by plans which provided (1) an adequate sample of the population studied, and (2) the use of several accredited technics for the examination of fecal samples.

MATERIAL AND METHODS

Population. Stools from 18,064 persons in or attached to the Air Force, Army, Marines and Navy were examined during the period 1952 to 1957 at four laboratories; 89% were active military personnel, 1% veterans, 3% dependents, and 7% food handlers with history of military service or out of state employment. The study group included patients and hospital employees, recent returnees from overseas, recent interstation transfers, auxiliary personnel and groups selected by barracks or job assignment, all stationed in Los Angeles, Riverside, San Bernardino or San Diego Counties. All 48 states were represented. The military persons had been in service 2 or more years and ranged in age from 24 to 56 years with the lower ages in preponderance. Approximately 30% had had overseas duty. Over 99% had lived outside of California. Approximately 80% had been stationed in 3 or more states. Approximately 12% were females. All military categories of rank and status were represented in ratios approximating those present at the different bases.

Collecting Materials. Laboratory procedures and facilities used in collecting and examining feces were the same at each of the four laboratories. Ambulatory persons delivered their own specimens in provided containers within minutes after defecation. Stools from bed patients were delivered by attendants within 1 hour. Specimens collected from nearby bases were transferred by car in 1 to 3 hours.

Smears and Examinations. Smears prepared by 12 or more technics were made from feces of different areas of each stool. Three wet smears were made using as diluent Locke-Ringer's Solution,[1] D'Antoni's Iodine[2] or Sudan black B.[3] Fixed smears included one or more stained with Heidenhain's iron-alum hematoxylin.[4] Other fixed smears included six or more prepared by Pearse's[5] cytochemical methods for carbohydrates, lipids, amino acids, calcium and iron, as part of another study. A smear from a zinc sulfate centrifugal flotation preparation was examined on each stool.[5] A De Rivas acid-ether centrifugation smear using approximately twice the weight of feces recommended was examined on 85% of stools. The latter proved particularly effective in concentrating cysts of *Giardia lamblia*, as well as *Entamoeba coli* and some ova.[6] All smears were examined systematically, the portion searched in each of the 12 or more slides being an area the length of the smear and the width of 10 low power fields. If identifications of organisms found in the first fecal specimens were doubtful, others were obtained. All identifications were made by the senior author.

RESULTS

Approximately one-fourth of the 18,064 stools examined (26.6%) contained one or more species of protozoa. The number per parasitized individual was 1.8. Two persons had 5 parasites each; 34 had 4 each. The most prevalent species were *Endolimax nana* (13%), *Giardia lamblia* (11.5%) and *Entamoeba coli* (10.6%). *E. histolytica* was detected in 2.9% of the persons examined, and *E. hartmanni* was found in 3.2% (Table 1). *Blastocystis hominis* was present in 17,089 of 18,064 persons or 94.6% prevalence. *Entamoeba polecki* was observed in one person.

The observed and estimated prevalences of *Giardia lamblia* by age groups of hosts are shown in Table 2. The heterogeneity X^2 (16.26) was

* Supported in part by a grant from Sigma Xi.

† San Bernardino County Health Department and Department of Biology, University of Southern California, Los Angeles. Present address 3956 Oakwood Place, Riverside, California.

‡ Biometrical Laboratory, University of California Citrus Experiment Station, Riverside, California.

TABLE 1

Prevalence of protozoa in 18,064 adults at military bases in southern California

Species	Number positive	% positive
Enteromonas hominis	3	<0.1
Embadomonas intestinalis	2	<0.1
Chilomastix mesnili	738	4.1
Giardia lamblia	2,077	11.5
Trichomonas hominis	113	0.6
Endolimax nana	2,382	13.2
Iodamoeba bütschlii	307	1.7
Dientamoeba fragilis	33	0.2
Entamoeba coli	1,908	10.6
*Entamoeba hartmanni**	572	3.2
Entamoeba histolytica	519	2.9
Total	8,654	26.6

* Diagnosis based on cysts less than 10 microns in diameter.

TABLE 2

Observed and estimated prevalence of Giardia lamblia *by age of host*

Age group	x*	No. of individuals		Prevalence	
		In sample	Positive	Observed	Estimated†
24–34	1	10,018	1,227	0.1225	0.1227
35–45	2	6,057	664	0.1096	0.1088
46–56	3	1,989	186	0.0935	0.0949
		18,064	2,077		

* Coded age-group (midpoint of age interval minus 18, divided by the interval length).

† $\hat{p} = a + bx = 0.1366 - 0.0139x$; $s_a = 0.0016$; $s_b = 0.0010$.

significant at the 0.001 level of probability, indicating that the prevalence was not independent of age, *i.e.*, the prevalence of infection was associated with age, becoming lower with increasing age. A weighted regression analysis resulted in the estimating equation: $\hat{p} = a + bx = 0.1366 - 0.0139x$, in which $\hat{p}$ is the estimated prevalence and x is the coded age group. Estimates of the standard errors are $s_a = 0.0016$, and $s_b = 0.0010$.

The other protozoa studied showed no significant differences in prevalence for the three age groups considered.

DISCUSSION

The sample appears to be sufficiently large and representative to establish confidence in the results of the survey. The heterogeneity of age, geography, race, sex and socioeconomics of the surveyed population appears to make the study-group as representative of the adult population of the United States as is readily obtainable. Strata not represented are those eliminated by military standards of selection. Most of the previous surveys have been based on institutional, local or highly restricted populations.[1, 5]

The raw data suggested some prevalence differences by age for the protozoa studied. Analysis of data on all species except the rare *E. hominis* and *E. intestinalis*, however, did not verify such differences in the age groups considered except for *G. lamblia*. Analysis of data on age groups covering a shorter span of years might have revealed additional age prevalence differences.

Many persons having *G. lamblia* were found positive only by concentration technics. The relatively low prevalence found by examination of wet and stained smears, as compared with that found by concentration of large fecal samples, may indicate that adults infected in childhood continue to harbor a decreased number of *G. lamblia*. In a number of instances cysts were very scanty even after concentration of the feces. The above determined prevalence, therefore, may approximate the higher actual prevalence.

SUMMARY

The intestinal Protozoa found in a sample of 18,064 adults stationed on or affiliated with military stations in southern California but representing all States and many different segments of the population in the United States were, in order of increasing prevalence (% in parenthesis), the following: *Embadomonas intestinalis* (<0.1); *Enteromonas hominis* (<0.1); *Dientamoeba fragilis* (0.2); *Trichomonas hominis* (0.6); *Iodamoeba bütschlii* (1.7); *Chilomastix mesnili* (4.1); *Entamoeba hartmanni* (3.2); *Entamoeba histolytica* (2.9); *Entamoeba coli* (10.6); *Giardia lamblia* (11.5); *Endolimax nana* (13.2). *Entamoeba polecki* was found in one individual only. Host age-groups 24–34, 35–45, 46–56 showed no significant changes in prevalence for any species except

Giardia lamblia, which decreased with increasing age.

REFERENCES

1. Belding, D. L., 1942. *Textbook of Clinical Parasitology*, 1st ed., p. 888. D. Appleton-Century Company, New York.
2. Jones, Ruth McClung, 1950. *McClung's Handbook of Microscopical Technique*, p. 790. Paul B. Hoeber, Inc., New York.
3. Pearse, A. G. E., 1953. *Histochemistry*, pp. 111, 112, 135–140, 146, 415–417. Little, Brown, and Company, Boston.
4. Chen, T. T., 1944. Staining nuclei and chromosomes in Protozoa. *Stain Technol.*, **19:** 83–90.
5. Craig, C. F., and Faust, E. C., 1951. *Clinical Parasitology*, 5th ed., p. 1032. Lea and Febiger, Philadelphia.
6. Brown, R. L., 1945. Evaluation of techniques used in the diagnosis of enterozoic parasitism in children. *J. Lab. & Clin. Med.*, **30:** 135–137.

ALTERATION OF THE ENVIRONMENT OF *ANOPHELES* LARVAE BY NUTRIA IN THE NAAMAN SWAMPS, ACRE DISTRICT, ISRAEL

S. EHRLICH AND D. SPIELBERG

Fisheries Division and Public Health Department, Haifa, Israel

The swamps and fish ponds extending 8 km along the Naaman River near Acre offer a serious malaria problem. During July, August and September, 1943, 2600 soldiers contracted malaria at a military camp in this area. Before the camp was established in 1942, the area was drained and a new bed was dug for the Naaman River (Figure 1). These precautions were effective during the first year when precipitation was 280 mm. Heavy precipitation (700 mm) in 1943 impaired the drainage, and the swamps were flooded. In 1944, as the precipitation was scant, the drainage system worked well and spraying by hand gave satisfactory results. Heavy rains in 1945 again flooded the swamps, which then had to be sprayed from the air with "malariol" and DDT. Since that time drainage has been poor and spraying from the air has been continued in the upper part of the Naaman course and in the spring swamps of this river. In the lower part, which is drained and cultivated, spraying by hand has been sufficient. The spraying is carried out 11 to 14 times a year with "malariol" + 2% DDT. Removal of water vegetation, formerly carried out in the whole area, is now limited to ditches in the drained areas which are sprayed by hand. In the swamps of the upper part of the Naaman, nutria have established themselves and are the only factor in clearing the water of weeds.

EFFECTS OF NUTRIA ACTIVITY

The nutria population of the Naaman area originates from 8 animals which in 1954 escaped from an unsuccessful nutria ranch at Kfar Massaryk near the lower northern end of the swamp area. The animals soon abandoned this improved section of the area and settled in the southern part, in the Afek fish ponds and adjacent Naaman swamps.

The improvement of the swamp area was not the only cause of this move. The animals could find near their ranch extensive, partly overgrown water surfaces in the Kfar Massaryk fish ponds. Probably the replacement of cattails (*Typha angustifolia*) and bulrushes (*Scirpus* sp.) by reeds (*Phragmites communis*) along the Naaman course, apparently brought about by an increase in the salinity of the river and adjacent swamps and ponds, induced the animals to settle in the fresher upper part where the water was overgrown by cattails and bulrushes.

In 1956, within 2 years after their escape, the nutria had cleared the uppermost part of the river. During 1957 to 1959 the cleared sector of the river was extended substantially. The animals alone kept all of the swamps directly adjacent to the fish ponds of Afek entirely clear of weeds. At the same time they destroyed cattails in the Afek fish ponds so that in 1959 only two small islands of cattails remained. As the cattails of the Afek fish ponds had formerly been cut for mat production and only ripe, full-grown cattails had been cut, leaving the rhizomes unharmed, there is no doubt that the complete removal of cattails from wide areas resulted from nutria activity.

After destroying the water vegetation, the nutria ranged into thickets along the banks of the Naaman River and adjacent ponds. Owing to the land plants' resistance to their grazing, the nutria have not destroyed their habitat and have remained in the area to serve as a permanent factor in keeping down the water vegetation.

Limitation of the vegetation-destroying activity of the animals to the flooded areas results from several causes. Grazing off the stalks beneath the water surface kills the plants, as water penetrates the opened sap arteries. On land moderate grazing on the stems only stimulates their regrowth. Moreover, the animals do not confine themselves to cutting off the stems of the emergent aquatic vegetation but dig out the rhizomes and roots as well (Figure 2). In this way they keep the water surface clear without damaging the land vegetation.

COMPARISON OF THE RESULTS OF NUTRIA ACTIVITY WITH OFTER METHODS OF REMOVING WATER WEEDS FROM STAGNANT WATER

The usual mechanical or chemical methods of removing emergent water vegetation from the

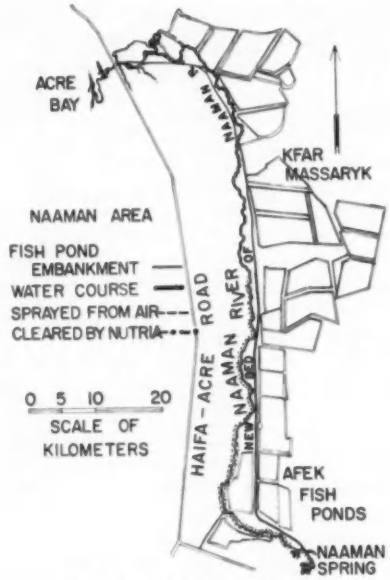


FIG. 1. The Naaman Valley

swamps and ponds lead to undesirable secondary effects. These methods clear larger surfaces at once. As long as the reeds shade the water surface, the development of algae is inhibited and the water beneath the plants remains clear. Thus, removal of the emergent water vegetation permits immediate illumination of the bottom. The dead stems lying on the bottom provide excellent attachment surfaces for the filamentous algae. Under such conditions a lush growth of filamentous algae sets in on the killed plants, soon covers the bottom and rises to the surface. This thick mat of floating algae lasts until August and provides excellent cover for the larvae of *Anopheles sacharovi* and some other mosquito species. The control or even the search for larvae becomes very difficult under such conditions.

Chemical killing of the filamentous algae with copper sulfate gives only transient results. The only satisfactory way to get rid of them is to remove them by hand, but this sometimes has to be repeated several times and is very expensive.

Unlike mechanical and chemical methods of removing the emergent vegetation, destruction by nutria in no case leads to the development of filamentous algae or any other floating vegetation. When the nutria population is small and scattered, it acts slowly on the emergent vegetation and from the open water edge only. Thus the unicellular algae dominating the open water penetrate the slowly expanding open area and by decreasing the transparency of the water prevent the development of filamentous algae.

A large, concentrated nutria population penetrates deeply into the beds of emergent weeds. There, in secluded spots, they increase the water turbidity while digging for the roots and rhizomes. This inhibits development of the filamentous algae on the bottom. Simultaneously, by manuring the water with their droppings, the animals stimulate the development of floating unicellular algae on the newly cleared spots, thus further preventing development of the filamentous types. The habit of leaving a shelter belt until the final destruction of a bed (Figure 3) keeps the cleared spots isolated from the main water body and saturated with manure. Thus, in the beds of cattails in the ponds of the Naaman area

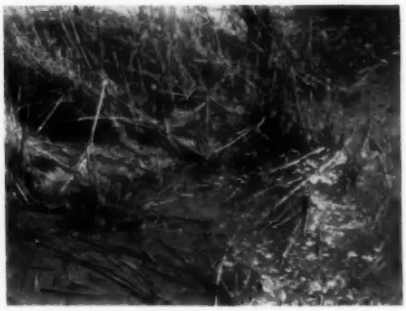


FIG. 2. Total destruction of reeds in the water, whereas on the bank they still remain. (Neve Eitan, Beth-Shean Valley).

FIG. 3. A shelter belt left until the end of depletion of a reed bed.

cleared by nutria, development of the undesirable filamentous algae has never occurred.

The same conditions have been established in the fenced ponds cleared by nutria in other areas of this country, such as the Nahsholim Fish Farm in the coastal area south of Haifa and in the Beth-Shean Valley, in the fish farms of Nir David, Messiloth, Neve Eitan and in the Kfar Ruppin Fish Farm in the Shmone pond. Only in a thick bed of reeds in the Vav pond at Kfar Ruppin has a transient strong development of filamentous algae occurred during destruction of the reeds by nutria. In this plot the development of reeds had been extremely strong and undisturbed for several years. Consequently, a heavy layer of dead vegetation had been deposited at the bottom. In the first phase of reed destruction the nutria, preferring the younger shoots of reeds to their tough rhizomes, did not disturb this dead layer. In this way conditons favorable to development of the filamentous algae were temporarily created. These algae clung first to the slightly immersed dead stalks and later developed into mats covering the water surface. However, as soon as the growing shoots were destroyed, the animals turned to the rhizomes, digging eagerly at the bottom, the layer of dead stalks with attached mats of algae was destroyed and the water became turbid; as the filamentous algae disappeared, the unicellular varieties occupied the entire pond.

Experience gained with nutria in various regions of this country under different conditions leads us to conclude that nutria can be considered as an effective agent for destroying emergent aquatic vegetation of this type. In every area inhabited by nutria the establishment of mats of horizontal vegetation providing cover and food for mosquito larvae has been prevented.

COMPARISON OF NUTRIA WITH OTHER METHODS OF KEEPING THE WATER COURSES CLEAN

The desirability of draining the swamp areas induced the local authorities to construct substantial and sometimes expensive drainage systems. However, underground drainage pipes were not effective, as they were quickly clogged with the roots of fast-growing, water-hungry subtropical bushes. Thus, underground drainage was abandoned; and for draining swamps in the Haifa Bay Area, south of the Naaman Area, an open drainage system of concrete-lined ditches was constructed. The draining of about 4 square kilometers cost £70,000 in 1929 to 1936, but the system proved to be ineffective. Filamentous algae clinging to the concrete plates lining the ditches clogged them very soon. Run-off from the swamps was stopped and the ditches themselves became dangerous mosquito - breeding places. They had to be continuously cleaned, as by the time the cleaning crew reached the end the starting point was again clogged. The concrete plates finally were removed and replaced by ordinary earth ditches.

For draining the swamps in the Naaman Area in the years 1942 to 1943, only earth ditches were used. In the larger ditches with rapid water-flow a lush growth of *Potamogeton* soon was established, furnishing food and cover for the larvae of *Anopheles hyrcanus* and *A. sacharovi*. The beds of *Potamogeton* gradually covered long stretches of the water courses, thereby impeding run-off

FIG. 4. Area of the Naaman bed cleared by nutria, showing temporarily undisturbed clumps with nests.

FIG. 5. Plant cover on the Naaman banks reaching to the water's edge.

from the swamps. This also occurred in sections of the Naaman River which had been mechanically cleared of reeds. The smaller ditches, without flow and with sticky bottoms, became overgrown with algal scum. The horizontal vegetation had to be continuously removed, as it soon reappeared.

On the other hand, the entire section of the Naaman which was cleared by nutria (Figure 1) during 1955 to 1957 remained completely free of horizontal vegetation (Figures 4, 5), up to the end of our observations in June, 1959. On the whole stretch cleared by nutria not a single plant of *Potamogeton* could be detected, notwithstanding the particularly heavy development of this plant in other sections of the Naaman drainage system in the spring of 1959, following an unusually mild winter.

This peculiarity of nutria action on stream vegetation in this area cannot be adequately explained. Comparable observations from other regions of this country are lacking, since nutria generally are bred only in stagnant water. Probably the undisturbed plant cover on the banks, which reaches to the water's edge, substantially prevents the erosion of the banks. The lack of deposition of eroded bank material in the bottom possibly could prevent the establishment of *Potamogeton* and other bottom attached plants. In such a situation only the invasion of perennial weeds by shoots and rhizomes extending from the banks could renew the plant cover. If the shoots descending beneath the water surface were destroyed by nutria, the water course would remain continuously clean. The presence of nutria in the bank vegetation in numbers which do not disturb the plant cover on the banks but suffice for the destruction of the emergent vegetation in the water would keep the water course clean.

If the density of nutria were to exceed the grazing resistance of the bank vegetation, erosion of the banks would result. This would carry silt from the banks to the bottom, thus creating conditions suitable for plants to take root. On the other hand, insufficient nutria density would allow the high swamp vegetation from the banks to overgrow the water course.

EFFECTS ON MALARIA CONTROL OPERATIONS

During the 3 years in which nutria kept down the water vegetation in the upper Naaman and adjacent ponds and swamps, the following effects on antimalaria measures were observed:

1. Consumption of insecticide was substantially decreased, as the pilot was able to see the water and could spray its surface only. In overgrown sections spraying of the entire valley was necessary.
2. Spraying efficiency increased since the insecticide was not retained on the leaves. This permitted a further decrease in the amount of insecticide used.
3. Exposure of the water course by the animals revealed the possibility of straightening the river bed and thus draining the adjacent swamps.
4. Destruction of the emergent vegetation by nutria was not followed by the development of the horizontal vegetation. Thus, the drainage capacity of the water courses was maintained and no new breeding sites for mosquito larvae were created.

SUMMARY

Various methods of removing reeds from a malaria infested region were compared as to their effectiveness in control of *Anopheles* larvae. It was found that the mechanical or chemical methods of reed destruction usually resulted in the development of horizontal vegetation which furnished shelter for mosquito larvae.

In contrast, a nutria population of suitable density was able to keep the river course and adjacent ponds clear of vegetation for 3 years. The results obtained with feral nutria in the Naaman swamps were confirmed in five fenced pond farms in various regions of the country.

In the area kept permanently clear of weeds by nutria the drainage capacity of the river and ditches was maintained, and the use of insecticide was decreased substantially.

SPONTANEOUS ACUTE TOXOPLASMOSIS IN A MARMOSET MONKEY*

KURT BENIRSCHKE AND RALPH RICHART

Departments of Pathology and Obstetrics, Harvard Medical School and the Boston Lying-in Hospital, Boston, Massachusetts

Spontaneous toxoplasmosis of monkeys has been recorded occasionally.[1] No such infection has been described in South American marmosets although some marmosets (*Marikina geoffroyi*) were readily infected by experimental administration of toxoplasmosis organisms.[2] It is the purpose of this paper to describe a fatal case of spontaneous toxoplasmosis in a cotton-topped marmoset (*Oedipomidas oedipus*). Large numbers of marmosets are imported into this country yearly, with a very high mortality rate, yet there are virtually no studies which attempt to explain the cause of death in these animals.

OBSERVATIONS

On 8/30/1959 an immature female marmoset (*Oedipomidas oedipus*) was received and introduced into a well-established breeding colony of marmosets. The animal had arrived in New York City via airplane from Colombia (South America) the previous day together with an immature male of the same species. The next day she was flown to Boston, the male remaining in New York. Both animals received one dose of oral tetracyclin (Achromycin®) upon arrival. The male animal died in New York within 3 days from "pneumonia". An autopsy was not performed.

The female was placed in a large cage together with a mature male who had lived in captivity for at least 3 years and had given rise to at least four pregnancies. His former mate had been sacrificed previously while pregnant. In three adjacent cages in the same room lived respectively three tamarins (*Tamarinus nigricollis*), five common marmosets (*Callithrix jacchus*) and two lion marmosets (*Leontocebus rosalia*).

The new animal arrived in seemingly excellent condition and soon began to play with her much senior partner. Her fur was not as glistening as that of adult animals but it was well groomed and of excellent density. The diet consisted of banana, grapes, cookies, mealworms and water or milk. Food was taken eagerly at first and the daily ultraviolet irradiation was enjoyed as much as it was by the others. The two partners groomed one another frequently as is seen only in compatible and healthy specimens and they spent the nights huddled together on a high branch in the room. There was no change on the morning of 8/31/59 but in the afternoon the female vomited once and seemed less eager to take food although she was still active and playful. On 9/1/59 she refused all food and took very little water. In the afternoon she was only slightly responsive, sitting in the warm sun of the window and having extremely rapid respirations (120–130/minute). She was then removed from the cage. One dose of tetracyclin (Achromycin®) was administered orally (125 mg). One hour later she expired with severe respiratory distress, foamy froth appearing at the nostrils terminally. The remaining monkeys have not shown any sign of illness to the time of this writing (12/25/59) despite the close contact with the affected female.

An autopsy was performed immediately. The monkey weighed 240 gm and had a snout to tip of tail length of 51 cm (Figure 1). In general there was no evidence of chronic illness; indeed there was a relatively large amount of subcutaneous and retroperitoneal fat. The following observations indicate immaturity of this animal: lack of epiphyseal fusion by x-ray, recently erupted last upper molar teeth, the low weight of an otherwise well-nourished animal and lack of Graafian follicles in the ovaries. The only pathological finding made on dissection was complete consolidation of the lungs. They were irregularly hemorrhagic and had a frothy cut surface. Bacterial cultures taken from the heart blood and the lungs remained sterile on blood agar and in broth as well as on Sabouraud's medium. A blood smear contained no microfilariae, an otherwise frequent finding in this species. No parasites were identified externally or in the gastrointestinal tract. Since it was assumed that the animal had died as a result of bacterial

* Supported by U. S. Public Health Grant RG 4966.

FIG. 1. Appearance of animal (*Oedipomidae oedipus*) at death. Overall length, 51 cm.

pneumonia, the central nervous system was not removed in order to keep the skeleton intact for dissection of the musculo-skeletal system. Radiologic examination of the bones was negative and there were no calcifications in the brain. The carcass was placed in a refrigerator at 0° C. The tissues were fixed in Bouin's solution.

Microscopic sections were made of all organs. Initially they were interpreted as showing pathologic changes of Chagas' disease. However, consultation with Dr. T. H. Weller and C. Pan (Department Tropical Medicine, Harvard Medical School) suggested *Toxoplasma gondii* as the probable responsible organism. Consequently small fragments of retroperitoneal muscle and one pelvic lymph node were removed from the cooled carcass on 9/4/59 and were homogenized with sterile sand; the supernatant then was injected intraperitoneally into five young mice. After 1 week two were sacrificed and an aliquot from a peritoneal lavage was inoculated into six further young mice. Five of the latter died within 5 days. The remaining mice were then sacrificed. Smears of the peritoneal surfaces and microscopic sections were made of all mice. Fresh wet preparations as well as Giemsa-stained smears showed typical toxoplasma organisms. The histologic changes in all mice were typical of toxoplasmosis. Control mice of the same colony remained healthy and two sacrificed animals contained no similar pathologic lesions. The strain of *Toxoplasma* obtained from this marmoset was not maintained any longer and no serologic studies were undertaken. For several reasons it was deemed inadvisable to attempt to bleed the other monkeys. The colony is primarily intended for breeding purposes and past experience has taught that the animals must be healthy and also "happy" in order to achieve results. Moreover, the drawing of blood from the femoral vessels of these animals in the past has been extremely difficult. Finally, the pathologic changes observed as well as the mouse inoculations were so typical of toxoplasmosis that the risk involved in obtaining accessory evidence seemed unjustified.

On microscopic examination of tissues from the marmoset the following pertinent changes were noted. Numerous intracellular clusters of organisms were present in the heart (Figure 2). For the most part they were not accompanied by inflammatory cells but in some areas they were attended by petechial hemorrhages and areas of necrosis. Reticulum stains (Wilder method) showed a very early pseudocyst wall. Typical pseudocysts as seen in chronic toxoplasmosis were not found. Giemsa stains failed consistently to show kinetoplasts for which reason a double infection with *Trypanosoma cruzi* was ruled out. One papillary muscle of the heart contained an irregular calcific mass with multinucleated giant cells. The cause of this lesion could not be determined. There was diffuse pulmonary edema (Figure 3) with scattered fresh hemorrhage. Within this fluid and in the alveolar walls numerous organisms could be found. There was

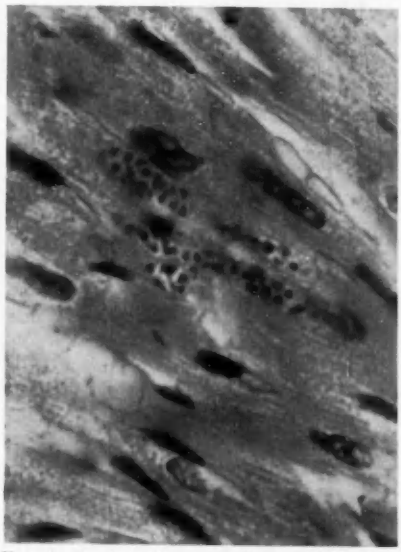


FIG. 2. Section of myocardium with two myocardial fibers containing organisms in the center. H&E × 900.

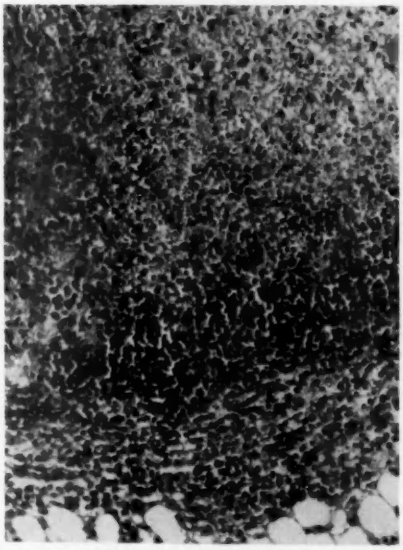


FIG. 4. Section of tracheo-bronchial lymph node with marked destruction (top) and infiltration of adjacent fat with inflammatory cells. H&E × 200.

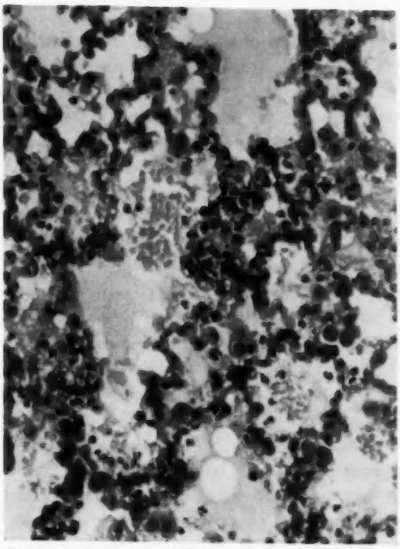


FIG. 3. Section of lung showing pulmonary edema, inflammatory reaction and fresh hemorrhage. H&E × 180.

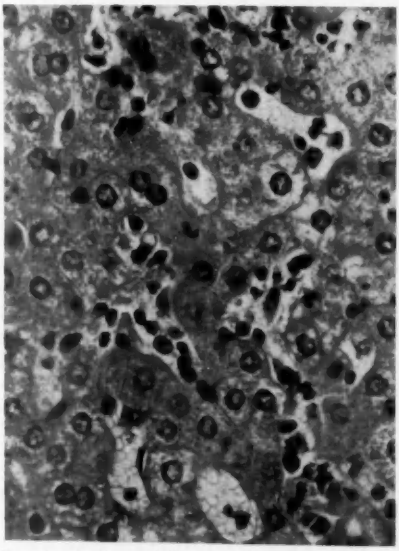


FIG. 5. Section of liver with diffuse inflammatory cell infiltration and degeneration. H&E × 400.

an early diffuse interstitial pneumonia. The spleen as well as most lymph nodes showed an acute inflammatory process with numerous *Toxoplasma* scattered throughout. The Malpighian follicles were markedly enlarged and some had necrotic central areas. The littoral cells were enlarged and were more numerous. One tracheobronchial lymph node was almost completely

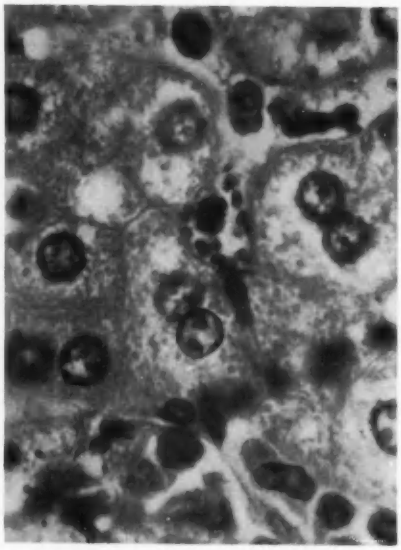


FIG. 6. Section of liver, toxoplasma are seen in several liver cells and the sinusoids. H&E × 900.

necrotic (Figure 4), contained numerous organisms and had many vascular occlusions by thrombus. Throughout the liver there were small foci of necrosis associated with petechial hemorrhage, early leukocytic response and many organisms (Figures 5, 6). Rarely an entire liver lobule was found to be necrotic. Similar degenerative lesions were present in the fasciculate zone of the adrenal glands and less commonly in the kidneys. Aggregates of organisms were present also in the diaphragm and other skeletal muscle and the bone marrow but there was no reaction in these tissues. Very small peritoneal exudative lesions also contained organisms. The gastrointestinal tract, skin, reproductive organs and pancreas showed no pathologic changes.

DISCUSSION

This observation appears to be the first record of spontaneous toxoplasmosis in a marmoset monkey. Recent reviews of this disease[1, 3, 4] have emphasized the widespread occurrence of this infection and it is not surprising that it should have been found in another species of South American monkeys.

The course of the disease in this monkey appears to have been an extremely rapid one. All lesions were of very recent nature, those in the lymph node and spleen being probably the oldest. It is estimated that the latter may have had their inception about 1 week before demise. The course thus resembles that described for the experimental infection of marmosets[2] and is quite similar to the course of toxoplasmosis in an epizootic in squirrel monkeys (*Saimiri sciurea*) at the Philadelphia Zoological Gardens.[5] Ruch[1] refers to subsequent fatal illnesses in monkeys due to *Toxoplasma* and occurring in the same animal house. As was the case in the present infection not all animals who were in contact with the diseased monkey experienced a similar illness. Serologic studies have not as yet been performed to ascertain whether this is due to existing antibodies in the apparently healthy animals or whether in fact these animals experienced an asymptomatic infection.

It has been considered that this marmoset may have had a double infection with *Toxoplasma gondii* and *Trypanosoma cruzi*. On morphologic grounds this possibility was excluded. Although many organisms in the heart were not attended by an inflammatory or degenerative lesion the histologic property of the organisms was identical with that of organisms in all other organs. The appearance of silver- and Giemsa-stained organisms invariably favored *Toxoplasma*. The histologic appearance of the organisms was compared with the criteria of Frenkel[6] for the differential diagnosis of several similar organisms and found to fit only *Toxoplasma*. Finally, the infection in the injected mice was so characteristic of this disease and the fresh wet preparations were so typical of *Toxoplasma* that we believe the death of this marmoset can only be explained on the basis of acute toxoplasmosis.

This report does not contribute to our imperfect knowledge of the mode of transmission of *Toxoplasma* in spontaneous infections. It must be surmised, however, that this animal and probably the male companion who died in New York simultaneously were infected within approximately 2 weeks before death and that this infection occurred after capture. These animals are generally held captive for some weeks in South America until order for shipment arrives from foreign countries. Since marmosets, and particularly *Oedipomidas oedipus* thrive on young rodents and birds one wonders if infection did not occur as the result of ingestion of infected rodents or birds, or insects. A majority of marmosets obtained for this colony have suc-

cumbed from infection with *Prosthenorchis elegans*, transmitted by ingested roaches at the animal dealers or zoos.

SUMMARY

A spontaneous infection with what appears to be *Toxoplasma gondii* of an immature *Oedipomidas oedipus* marmoset is reported. The animal arrived five days prior to death from Colombia and had an overwhelming infection with death due to interstitial pneumonia and pulmonary edema. Organisms identified as *Toxoplasma* on the basis of morphology and transmissibility to mice were recovered from the carcass and were found in all major organs.

REFERENCES

1. Ruch, T. C., 1959. *Diseases of Laboratory Primates*. W. B. Saunders Co., Philadelphia.
2. De Rodaniche, E., 1954. Susceptibility of the marmoset, *Marikina geoffroyi*, and the night monkey, *Aotus zonalis*, to experimental infection with *Toxoplasma*. *Am. J. Trop. Med. & Hyg.*, **3:** 1026–1032.
3. Frenkel, J. K., and Friedlander, S., 1951. *Toxoplasmosis*. Public Health Service Publication No. 141, Washington, D. C.
4. Jacobs, L., 1956. Propagation, morphology and biology of *Toxoplasma*. *Ann. New York Acad. Sc.*, **64:** 154–179.
5. Ratcliffe, H. L., and Worth, C. B., 1951. Toxoplasmosis of captive wild birds and mammals. *Am. J. Path.*, **27:** 655–667.
6. Frenkel, J. K., 1956. Pathogenesis of toxoplasmosis and of infections with organisms resembling *Toxoplasma*. *Ann. New York Acad. Sc.*, **64:** 215–251.

A STUDY OF ASCARIASIS ERADICATION BY REPEATED MASS TREATMENT

FRANCISCO BIAGI F. AND OSCAR RODRIGUEZ

Unidad de Patologia, Escuela de Medicina, U. N. A. M., Hospital General, Mexico 7, D. F.

In areas where ascariasis is common, intestinal obstruction and perforation caused by it are well known complications, frequently leading to hospitalization, surgical intervention and death.[1-4] Moreover, *Ascaris* infection probably is an important factor in the malnutrition of some populations.[5]

Ascariasis is acquired mainly, if not entirely, from infected members of the human population. Morphological[6] and serological[7] differences between *Ascaris lumbricoides* and *A. l. suum* have been described; and although transitory mature experimental infections with pig *Ascaris* have been reported,[8] there is no evidence that pigs are significantly involved in the epidemiology of human ascariasis. Environmental conditions, such as low humidity or high temperature, kill *Ascaris* eggs in a short time.[9] Beaver[10] has found that viable *Ascaris* eggs disappear rapidly in sandy soil during the dry season. In our laboratory these eggs lose their infectivity after approximately 6 months when maintained in 2% formalin at 28°C. Under more favorable conditions they might live longer, but such conditions for prolonged periods generally do not occur in nature.

In the treatment of ascariasis, piperazine has been demonstrated to be a very useful drug. It is highly effective, inexpensive, palatable and relatively non-toxic. It has been successfully used in single-dose mass treatments.[11] Total removal of the infection from a population, although transitory, can be expected with repeated mass treatments.

If mass treatment were repeated every 30 days, the recently acquired *Ascaris* would be destroyed before reaching maturity. As a result, additions of *Ascaris* eggs to the soil would be avoided; and in due course, even with continuous fecal contamination, infective eggs would disappear from the environment. Thus, the biological cycle of *A. lumbricoides* having been broken, the species would disappear. Several conditions could contribute to failures in a program of this type, viz., new infections acquired from pigs, residual infections remaining in the human population, persistence of eggs in the soil beyond the period of repeated mass treatments, and reintroduction of new infections through the movement of persons in and out of the control area.

The present study was designed to determine the practicability of eradication of ascariasis from a small population by means of single-dose administrations of piperazine repeated monthly.

MATERIALS AND METHODS

The present study was done in Astapa, a small village in the State of Tabasco, Mexico. Astapa is located in a tropical region, with annual average temperature of 25.4°C and annual precipitation of 2572 mm. It has 529 inhabitants, living in 89 houses, the majority of which are made of wood with palm roofs and earth floors, and lack tap water, drainage, electricity and sanitary facilities. Defecation directly on the soil within the village is a common habit. One or several pigs are associated with almost every household. According to local butchers, worms are seldom found in these animals. A program of repeated monthly administration of an antiparasitic drug was proposed to the local authorities and citizens of Astapa, and enthusiastic collaboration was obtained.

In a palatable syrup containing 10 g of piperazine hexahydrate and 200 mg of Superinone, a surfactant, per 100 ml,* piperazine hexahydrate was administered in a single dose of 100 mg/kg body weight, not exceeding 4 grams total for the single dose. Treatments were given simultaneously once a month to all individuals for 8 consecutive months (February to September). Due to temporary absences, sickness, or other causes, 5 to 8% of the population on each occasion missed taking the drug. Six persons (1.1%) did not participate.

Data on the number and size of the worms expelled from individuals of different age groups were recorded. No sanitary measures were applied, and no education was attempted.

RESULTS

In all, 3,968 individual treatments were given. There were no untoward side-effects or incidents.

* Bryrel with Superinone, Winthrop Products.

After the first treatment, 115 persons (28.3%) expelled *Ascaris*. As in other places, infections were more prevalent in children than in adults (Table 1). With the first mass treatment, 519 *Ascaris* were eliminated. With the second, 205 immature and 14 mature worms were expelled. The immature ones were those which had come into the intestine during the interval between the two treatments, indicating with some accuracy the rate of acquisition of infection in Astapa under "normal" conditions. The 14 mature *Ascaris*, expelled in the second treatment, can be considered as having escaped or resisted the first treatment. They represented only 2.7% of the total initially existing in the village. Thus the first treatment eliminated 97.3% of the worms.

The number of persons who expelled worms and the number of worms eliminated diminished each month. Also, with each successive treatment there was an increase in the proportion of immature worms among those passed. Conversely, the number of mature worms remaining in the population, and thus the amount of infectivity added to the environment, was rapidly reduced after the first treatment; no mature worms remained after the sixth treatment (Table 2).

TABLE 1

Frequency of ascariasis by age groups in Astapa as revealed by results of the first treatment

Age in years	Persons in group		
	Total	Infected	
		No.	%
0–1	35	2	5.7
2–6	90	35	38.9
7–14	124	39	31.4
15–29	143	23	16.1
30+	137	16	11.7
Total	529	115	28.3

TABLE 2

Number and frequency of worms removed from 529 individuals by eight successive treatments given monthly

Treatment no.	Persons passing worms		Number of worms passed		
	No.	%	Total	Immature	per 100 persons
1	115	28	519		98
2	63	12	219	205	41
3	40	8	141	138	27
4	16	3	43	22	8
5	13	2	46	40	9
6	12	2	48	45	9
7	2	1	3	3	1
8	0	0	0	0	0

DISCUSSION

In this experiment the biological cycle of *Ascaris* was broken, and it was demonstrated that by monthly mass treatments with the piperazine mixture employed, the addition of eggs to the soil can be decreased rapidly. Thus, depending upon the climatic conditions and character of the soil, the infectivity of the environment similarly can be reduced to a negligible level within a few months. However, to achieve complete and lasting absence of ascariasis in a given population would require extensive programs simultaneously and effectively carried out in adjacent areas. Before undertaking such a large-scale program, it would be necessary to make an objective evaluation of the "costs of the parasitosis" and the "price of its eradication", the latter being possible only after determining the rates of development and survival of *Ascaris* eggs in different soils, seasons and climates. Nevertheless, in view of the possibility of controlling contamination and essentially eliminating the infectivity of the soil, ascariasis may be regarded as an eradicable human disease.

It is significant to note that a total of 3,968 single-dose treatments with piperazine resulted in desirable effects only.

SUMMARY

In a village of 529 inhabitants in southeastern Mexico, mass treatments using piperazine in a single-dose, repeated monthly, reduced the ascariasis rate from 28% at the first treatment to no detectable infection at the eighth. Mature worms capable of contributing infectivity to the soil were not seen in post-treatment stools after the sixth month. It is concluded that eradication of human ascariasis by repeated mass treatments can be achieved, at least in a closed population.

REFERENCES

1. Fernando, P. B., and Balasingham, S., 1943. Acute ascariasis in children. *Indian J. Pediat.*, **10**: 149–173.
2. Cespedes, R., 1953. Ascaridiosis hepatica y de

las vias biliares. Estudio de cinco casos anatomo-clinicos. *Rev. Biol. Trop.*, **1:** 197–221.

3. DE LA TORRE, J., AND ARREDONDO, L., 1954. Complicaciones quirurgicas abdominales de la ascaridiasis en el nino. *Bol. Med. Hosp. Inf. Mex.*, **11:** 143–170.
4. GOMEZ, F., 1959. *Escenas de Hospital*, ser. III. Mexico, D. F.
5. VENKATACHALAM, P. S., AND PATWARDHAN, V. N., 1953. The role of *Ascaris lumbricoides* in the nutrition of the host. Effect of ascariasis on digestion of protein. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **47:** 169–175.
6. SPRENT, J. F. A., 1952. Anatomical distinction between human and pig strains of *Ascaris*. *Nature*, **170:** 627.
7. BIAGI, F., F., PAYAN, A. M., CUENCA, G., AND HERNANDEZ-CHINAS, J., 1959. Valor de una intradermo-reaccion y una reaccion de precipitacion en el diagnostico de la ascariasis. To be published.
8. TAKATA, I., 1951. Experimental infection of man with *Ascaris* of man and the pig. *Kitasato Arch. Exper. Med.*, **23:** 49–59.
9. FANTONI, V. J., 1946. Estudio experimental de algunos factores que pueden influenciar el desarrollo de los huevos de *Ascaris lumbricoides* en nuestro medio. *Rev. méd. Rosario*, **35:** 16.
10. BEAVER, P. C., 1952. Observations on the epidemiology of ascariasis in a region of high hookworm endemicity. *J. Parasitol.*, **38:** 445–453.
11. ATCHLEY, F. O., WYSHAM, D. N., AND HEMPHILL, E. C., 1956. Mass treatment of ascariasis with a single dose of piperazine citrate. *Am. J. Trop. Med. & Hyg.*, **5:** 881–887.

THE EFFECT OF METABOLITES ON THE ANTITOXOPLASMIC ACTION OF PYRIMETHAMINE AND SULFADIAZINE

DON E. EYLES AND NELL COLEMAN

*Laboratory of Parasitic Chemotherapy, National Institute of Allergy and Infectious Diseases**

Studies from this laboratory and others have shown that a strong synergistic action against *Toxoplasma gondii* takes place when pyrimethamine (Daraprim®) and sulfonamides are used jointly in the treatment of this infection.[1] This finding, taken in conjunction with studies on malaria and in vitro studies with bacteria, indicates that the locus of action is almost certainly in the *p*-aminobenzoic acid-folic-folinic acid area, and is possibly due to imposing sequential blocks upon a single metabolic pathway.[2]

Summers[3] has shown that *p*-aminobenzoic acid (*p*ABA) and folic acid antagonized the effect of sodium sulfathiazole against mouse toxoplasmosis. Summers[4] also found that folic acid did not interfere with the antitoxoplasmic action of pyrimethamine in mice when used alone. Frenkel and Hitchings,[5] also using mice, added confirmatory information and presented new evidence on the effects of *p*ABA, folic and folinic acid on the antitoxoplasmic effect of pyrimethamine and sulfadiazine, alone and in combination. A broad background of information on similar relationships in the chemotherapy of malaria also exists.[2]

These previous findings point toward the possibility of using metabolite-antimetabolite studies for further elucidation of the mode of action of these drugs. In addition to the fundamental aspects of the study, there also exists the possibility that metabolites might be used to counteract the toxicity of drugs in man. Pyrimethamine with sulfonamides at the dosages used at present in man for the treatment of toxoplasmosis has produced toxicity reactions in some human subjects. There is evidence from malaria studies and from the studies of Summers, Frenkel and Hitchings that some of the metabolites do not necessarily interfere with the antiparasitic action of the drugs.

The present paper reports work directed toward the above ends. It confirms in part the work of Frenkel and Hitchings and supplements it by providing more completely quantitative information on the metabolite-antimetabolite relationships.

* Located at the University of Tennessee, School of Medicine, 874 Union Avenue, Memphis 3, Tennessee.

MATERIALS AND METHODS

All experiments were done with the Norway rat strain of *Toxoplasma gondii*. The same strain has been used extensively by this laboratory in previous studies on chemotherapy.[6] It is a very virulent strain which invariably produces death in untreated mice, even when the inoculum is reduced to a few organisms.

The mice used in the study were of the NIH general purpose strain and weighed about 20 grams each. The mice were inoculated intraperitoneally with 20,000 *Toxoplasma* organisms obtained from the peritoneal exudate of infected mice, the number being estimated by counting in a hemacytometer. Physiological saline was used as the diluent to obtain the requisite number of organisms in 0.1 ml of inoculum. Untreated control mice usually died 6 to 8 days after inoculation.

Drugs and metabolites were incorporated in ground Purina Laboratory Chow and offered to the mice *ad libitum* just after inoculation. Treatment was continued for 14 days. Dosages are expressed as percentages of drug in diet (in mg%). These may be converted to mg/kg/day by multiplying by a factor of 2, this being based upon 4 grams of food per day, the approximate amount consumed by our mice.

The testing of the metabolites-antimetabolite relationship was approached in two ways. In the first method, arbitrary drug dosages higher than the median effective curative dosage were administered; then, the proportion of metabolite was varied and the effect measured in terms of time to death and proportion of mice surviving.

The effects of the metabolites were also assessed in some instances by determining the dose-effect curves of the drugs with and without metabolite. This latter we consider to be a very sensitive indicator of effect, as any shift of the dose-effect curve due to the use of the metabolite

is easily detectable and subject to statistical testing. Drug and metabolite regimens are given in the results section.

Any mice dying during the course of the experiments were examined carefully for *Toxoplasma* organisms. Mice surviving for 42 days were sacrificed and tissues passed to uninfected mice to determine if cure had been obtained, except that during part of the study this subinoculation procedure was not followed because of a shortage of mice.

All deaths of mice during the experiments from the 6th through the 42nd day were attributed to toxoplasmosis even though parasites were not always found on autopsy. This conservative procedure led to the inclusion of some mice which died of other causes.

Pyrimethamine for the study was furnished to us through the courtesy of Dr. George H. Hitchings of the Wellcome Research Laboratories. Folic and folinic acids were very kindly furnished by Dr. James M. Smith of Lederle Laboratories. Other substances were obtained from commercial sources.

RESULTS

The effect of p-aminobenzoic acid on the action of pyrimethamine and sulfadiazine, alone and in combination. In the first group of experiments a uniform dosage of 32 mg % of pyrimethamine was given in the diet to mice with and without varying proportions of *p*ABA. The dosage of 32 mg % is about 1.5 to 2 times the median effective curative dosage as measured upon a number of occasions in this and previous studies. *p*ABA was used in dosages varying from 32 to 512 mg % and the results of the experiment are presented in table 1.

The lowest dosage of *p*ABA (32 mg %) significantly reduced the proportion of mice ultimately cured and slightly reduced the proportion surviving through the treatment period of 14 days. On the other hand, the tremendous dose of 512 mg% of *p*ABA did not completely antagonize the effect of the pyrimethamine. One of 12 mice was cured in spite of the metabolite, and times to death of the mice which did not survive were 3 to 5 days longer than the times to death of the untreated control mice.

Based upon the observation that the curative effect of 32 mg % pyrimethamine was reduced drastically by an equal dose of *p*ABA, an experiment was undertaken to determine the extent to which an equal amount of *p*ABA would shift the median effective curative dosage (MED) of pyrimethamine. Under the conditions of our experiments this had varied from 12.5 to 22.5 mg% in the diet. Table 2 (first two columns) shows that the addition of an equal amount of the vitamin raised the MED to close to 64 mg% in the diet, or nearly 3 to 5 times the dosage without *p*ABA.

The next group of experiments was designed to verify the report of others that *p*ABA inhibits the antitoxoplasmic action of sulfonamides. A curative dosage of 125 mg% sulfadiazine in the diet was given to groups of 10 to 24 mice. The *p*ABA dosage was varied from 0 to 250 mg % in the diet. Results are summarized in Table 3.

Without *p*ABA nearly all mice were cured in

TABLE 1

Effect of p-*aminobenzoic acid on the antitoxoplasmic effect of pyrimethamine, all mice being given 32 mg % pyrimethamine in the diet, with the concentration of* p*ABA being varied and treatment continued for 14 days following inoculation with 20,000* Toxoplasma *organisms*

Mg % *p*ABA in diet	No. of mice	Day of death*	% surviving 14 days	% cured
0	12	11, 12, **15**	83	75
32	24	8, 11, 12, **12**, 13, **14**, 14, 14, **15**, **15**, **16**, 17, **19**, **19**, **19**, 21, **21**, 21	67	25
64	12	8, 12, **14**, **14**, 14, **14**, **16**, 18, 19, **19**, 20	50	8
128	12	10, **11**, **12**, **12**, 12, 12, **13**, **13**, 13, 14, 15	17	8
256	12	10, 11, **11**, 12, 12, **13**, **13**, **13**, 13, 15, **16**, **26**	25	0
512	12	10, 10, **11**, **11**, 11, **12**, **12**, **12**, **12**, **12**, **13**	8	8
N.T.†	12	**7**, **7**, **7**, **7**, **7**, **7**, **7**, **7**, **7**, **7**, **8**, **8**	0	0

* Bold face numeral indicates that *Toxoplasma* were seen at autopsy; however, the conservative assumption is made that all deaths were due to this infection. Same notation is used on subsequent tables.

† No treatment given.

TABLE 2

Dose-effect relationship for pyrimethamine as influenced by the concomitant administration of p*-aminobenzoic, folic and folinic acids*

Mg % drug in diet	No metabolites added			With equal amt. ƥABA			With 2 × amt. folic			With ¼ × folinic acid		
	No. of Mice		% cured†	No. of mice		% cured†	No. of mice		% cured†	No. of mice		% cured†
	used	cured		used	cured		used	cured		used	cured	
64	12	10	83	9	5	56	12	11	92	12	12	100
32	11	8	73	24	8	33	12	8	67	12	9	75
16	10	9	90	12	0	0	11	6	55	12	1	8
8	10	0	0	12	0	0	12	2	17	12	0	0
MED‡ = about 12.5 mg % in the diet*				MED = about 60 mg % in diet			MED = about 14 mg % in diet			MED = about 26 mg % in diet		

* In other experiments the MED for pyrimethamine varied from 12.5 to 22.5 mg %.
† As determined by subinoculation.
‡ Median effective dose.

TABLE 3

The effect of p*ABA on the action of a curative dosage of sulfadiazine (125 mg % in the diet), the treatment period being 14 days*

Mg % ƥABA in diet	No. of mice	Day of death of mice	% surviving 14 days	% cured†
0	23	6	96	96
16	10	16, 22, 24, 25, 26, 34	100	40*
125	11	7, 7, 7, 7, 8, 8, 8, 9, 9, 18, 21	18	0
250	24	7, 7, 7, 7, 7, 8, 8, 8, 8, 8, 8, 8, 9, 9, 9, 9, 9, 9, 10, 10, 10, 10, 12, 43	4	0

* Survival rate for 36 days rather than proportion cured.
† As determined by subinoculation.

the present experiments. When 16 mg % of *p*ABA was used, there was no immediate effect but the number of mice surviving 36 days was much reduced. When 250 mg % of *p*ABA was used, most mice died within 10 days. The sole long term survivor was found to be infected when sacrificed. Comparison with untreated mice indicated that this dosage of *p*ABA almost completely antagonized the effect of the sulfonamide.

As the experiments had established that *p*ABA antagonized the effect of both sulfadiazine and pyrimethamine when used alone, similar experiments were carried out using the two drugs in combination.

The first experiments used the dosage of drugs previously employed by Frenkel and Hitchings;[5] that is, 30 mg% of each drug in the diet. The *p*ABA dosage was varied from 30 to 400 mg % in the diet. As will be noted in Table 4, there was no significant effect upon 42 day survival even with the highest dosage of *p*ABA.

This experiment was done during a period of mouse shortage, so no subinoculations were done to see if survivors were free of parasites; however, dye tests were run on serum from the survivors with very interesting results. As is shown in the last column of Table 4, none of the mice receiving no *p*ABA or the lowest dose of 100 mg % in the diet had dye tests indicative of infection. On the other hand, a third of the mice receiving 200 mg % *p*ABA had dye test of 1:1000 or higher indicating cure had not been achieved.

Since the dosage of the drugs employed was many times greater than that necessary to cure toxoplasmosis in mice,[1] we considered that a more precise judgment of the influence of *p*ABA might

be made by determining the effect of the concurrent administration of this metabolite on the dose-effect relationship. Table 5 summarizes the experiment (this table also presents information on folic and folinic acids which will be discussed in another section of this paper). A mixture of drugs consisting of 80 parts of sulfadiazine to 20 parts of pyrimethamine was used, this being the same combination used in previous experiments on synergistic treatment. When twice the amount

TABLE 4

Effect of p*ABA on a mixture of sulfadiazine and pyrimethamine (30 mg % in the diet of each drug), the treatment period being 14 days*

Mg % pABA in diet	No. of mice	Day of death of mice	% surviving 14 days	% surviving 42 days	% dye test positive
0	12		100	100	0
100	12	34	100	92	0
200	12		100	100	33
400	12	16	100	92	100
No RX		5, 5, 5, 6, 6, 6, 6, 6, 6, 7, 7, 7	0	0	

TABLE 5

Effect of p*-aminobenzoic acid, folic acid, and folinic acid on the action of sulfadiazine and pyrimethamine against toxoplasmosis in mice, the treatment period being 14 days*

Dosage in mg % in diet		No. of mice	% surviving 14 days	% cured†	Approximate MED‡
pyrimethamine and sulfadiazine*	metabolite				
64	none	12	83	67	6 mg %
32		12	92	75	
16		12	92	67	
8		12	92	92	
4		12	33	0	
	pABA				
64	128	11	100	50	64 mg %
32	64	11	100	18	
16	32	12	100	8	
8	16	10	50	0	
4	8	10	0	0	
	folic acid				
64	128	11	100	91	6 mg %
32	64	11	100	100	
16	32	12	92	92	
8	16	12	92	75	
4	8	11	45	9	
	folinic acid				
64	16	12	100	100	11 mg %
32	8	12	100	100	
16	4	12	100	100	
8	2	12	83	33	
4	1	12	17	0	

* Mixture was sulfadiazine 80 parts and pyrimethamine 20 parts.

† As determined by subinoculation.

‡ Median effective dose.

of *p*ABA was used, the MED was raised from 6 mg % in the diet for the mixture alone to about 64 mg % in the diet when *p*ABA was added. Thus, the addition of the vitamin had a small but definite antagonistic effect on treatment with the combination of drugs.

The effect of folic acid. Varying proportions of folic acid were administered concurrently with a dosage of 32 mg % pyrimethamine in the diet. As stated above, this dosage of pyrimethamine is about 1.5 to 2 times the MED. It can be seen from Table 6 that even the highest dosage of folic acid did not antagonize the effect of the pyrimethamine.

Unfortunately subinoculation of tissue from the 57-day survivor could not be done, but it is most likely in view of the long survival period that parasite cure was achieved.

Another experiment was set up in which the effect of folic acid on the dose-effect curve of pyrimethamine was determined. This experiment is summarized in Table 2, and it can be seen that an amount of folic acid twice that of the drug did not alter the dose effect relationship. In this experiment parasite cure was demonstrated by subinoculation.

In contrast, folic acid did partially antagonize the effect of 125 mg % sulfadiazine although immediate survival was not affected. Progressively poorer long term survival was noted as the folic acid dosage was increased to 62.5 mg %; but even with this highest dosage, the inhibition of drug effect was only partial (table 7).

Having determined that folic acid did not interfere with the action of pyrimethamine but did incompletely antagonize the effect of sulfadiazine, we designed an experiment to determine

TABLE 6

Effect of folic acid on the action of a curative dosage of pyrimethamine (32 mg%), the period of drug administration being 14 days

Mg % folic acid in diet	No. of mice	Day of death of mice	% surviving	
			14 days	57 days
0	10	10, 34	90	80
16	12		100	100
32	11	6, 13	82	82
64	9	7, 7	78	78
No RX	12	**5, 6, 6, 6, 6, 7, 7, 7, 7, 8, 8, 8**	0	0

TABLE 7

The effect of folic acid upon a curative dosage of sulfadiazine (125 mg% in the diet), the period of drug administration being 14 days

Mg % folic acid in diet	No. of mice	Day of death of mice	% surviving	
			14 days	57 days
0	12	**27**	100	92
16	12	11, 20, 28	93	75
31	12	**24**, 25, 32, 32, 33	100	58
62.5	11	23, **25**, 26, **28**, 30, **32**, 33	100	36
No RX	12	**5, 6, 6, 6, 6, 7, 7, 7, 7, 8, 8, 8**	0	0

the effect of this vitamin on the dose-effect relationship of a mixture of the drugs. The amount of folic acid used was 2 times the total amount of pyrimethamine and sulfadiazine in the diet. This experiment is summarized in Table 5.

The median effective dosage with and without folic acid was essentially the same, being on the order of 6 mg % in the diet. Thus, a rather large dosage of folic acid had no inhibitory effect on the action of the combination of drugs, and at higher drug dosages the effect would appear to be enhanced by the vitamin, this possibly being due to counteraction of the toxic effects of the drugs.

The effect of folinic acid. The amount of folinic acid available to us was limited, and thus restricted the work we were able to do with it. Our principal effort was to determine the effect upon the dose-effect relationships of the drugs alone and the synergistic combination.

First, a dose-effect curve was run with pyrimethamine with a one-fourth amount of folinic acid added as is shown in Table 2. This gave a median effective dosage of 26 mg % in the diet with the added vitamin. This figure is not strictly comparable with the MED of 12.5 for pyrimethamine alone, shown in the table, as the two MED's were determined in experiments at different times. On other occasions the MED of pyrimethamine was as high as 22.5 mg % in the diet, so it can be concluded that the effect of the dosage of folinic acid used was either nil or very slight.

Similarly, a dose-effect curve for sulfadiazine was calculated with and without a one-eighth amount of folinic acid. The data obtained (not tabulated) did not indicate any shift in the median effective dosage.

Finally a dose-effect experiment, the results of which are summarized in Table 5, was run using a mixture of the two drugs. In this case, the MED was approximately twice the figure for the mixture alone, but this difference is of doubtful significance. Certainly the effect of the folinic acid was very slight.

In the dose-effect experiment attention should be called to the 100% cure rates in the groups given the highest dosages of folinic acid. As stated in the introduction, we followed the conservative procedure of attributing all mouse deaths to toxoplasmosis, so that any deaths in which drug toxicity played a role would appear in our tables as deaths from toxoplasmosis. It is likely that folinic acid produced the 100% survival through an effect on the toxicity of the drugs.

DISCUSSION

The studies we have made confirm those of others so far as the effect of *p*-aminobenzoic acid upon the antitoxoplasmic activity of sulfonamides is concerned; that is, the vitamin completely antagonized the effect of the drug when used at a level near that of the drug. This is in accord with all that is known about the action of sulfonamides.

Similarly, we confirmed the observation that *p*ABA antagonizes the effect of pyrimethamine;[5] however, our data show that the antagonism was incomplete. Some mice were cured and survival was prolonged with even the highest dosages of *p*ABA.

Likewise, *p*ABA antagonized the action of the two drugs in synergistic combination. This finding is contrary to that of Frenkel and Hitchings,[5] who stated that *p*ABA in mice "failed to alter the chemotherapeutic action of the combination"; however, the table of these authors shows a suggestion of antagonism. Their dosage of the combined drugs was much higher than was usually necessary for cure, thus possibly masking the effect of the *p*ABA.

Folic acid antagonized the effect of sulfadiazine incompletely, as expected. It did not antagonize the action of pyrimethamine. It did not shift the dose-effect curve of the two drugs in combination. If the hypothesis of Frenkel and Hitchings that the degradation of folic acid with the liberation of *p*ABA is the explanation of the antagonism of the sulfonamide, then the liberated *p*ABA should have shown some effect upon pyrimethamine. Likewise, the liberated *p*ABA should have produced a shift in the dose-effect curve of the drugs in combination.

As our supply was limited, we could not use folinic acid to the extent we wished. We were able to show that at a dosage one-fourth that of pyrimethamine, the dose-effect relationship was little if at all changed. A one-eighth amount with sulfadiazine did not alter the dose-effect curve. A dosage one-fourth that of the synergistic combination resulted in a slight increase in the amount of drug necessary for median effect, but the change is of doubtful significance.

All in all, the effect of folinic acid was either nil or very slight. The dosage we used was probably in excess of that necessary to reverse toxic effects of pyrimethamine. With high drug dosages folinic acid seemed to enhance the effect of the drugs. Most likely this was due to an effect on toxicity of the drugs at the high dosages.

The most complete theoretical discussion of the effects of the antitoxoplasmic drugs was presented by Frenkel and Hitchings.[5] The findings of our study for the most part are theoretically satisfied by the model set up by them, with the possible exception of the strong antagonistic effect of *p*ABA toward pyrimethamine used alone.

So far as the implications of the study for the treatment of toxoplasmosis are concerned, it is evident that in the mouse neither folic nor folinic acid interferes materially with the antitoxoplasmic action of pyrimethamine and sulfadiazine in combination. Frenkel and Hitchings[5] showed that folinic acid prevented or relieved toxic effects of pyrimethamine. They suggested that folic acid may have a similar effect and called attention to one of their patients who showed no signs of pyrimethamine toxicity while taking a vitamin preparation containing folic acid. On the other hand, Hitchings[7] presented evidence that in rats folic acid did not reverse pyrimethamine toxicity. If folic acid does prevent pyrimethamine toxicity in man, then it might well be used to increase the therapeutic efficiency of the pyrimethamine-sulfadiazine combination; but more information on the effect of folic acid is needed.

SUMMARY

Studies of the inhibition by various metabolites of the antitoxoplasmic action of pyrimethamine and sulfadiazine in acute mouse toxoplasmosis, alone and in combination, resulted in the following findings:

1. *p*-Aminobenzoic acid completely antagonized the effect of sulfadiazine and partially antagonized the effect of pyrimethamine. It also partially inhibited the effect of the drugs in combination.

2. Folic acid incompletely antagonized the effect of sulfadiazine, but did not antagonize the effect of pyrimethamine. It also had no effect upon the drugs when given in combination.

3. Folinic acid did not materially antagonize the action of the combination of pyrimethamine and sulfadiazine or of the drugs individually.

REFERENCES

1. Eyles, D. E., and Coleman, N., 1955. An evaluation of the curative effects of pyrimethamine and sulfadiazine, alone and in combination, on experimental mouse toxoplasmosis. *Antibiotics & Chemother.*, **5**: 529–539.
2. Goodwin, L. G., 1956. Sulphonamides and folic acid antagonists in malaria and toxoplasmosis. *Proc. Roy. Soc. Med.*, **49**: 871–873.
3. Summers, W. A., 1947. Antagonism of sulfonamide inhibition by paraaminobenzoic acid and folic acid in *Toxoplasma* infected mice. *Proc. Soc. Exper. Biol. & Med.*, **66**: 509–511.
4. Summers, W. A., 1953. The chemotherapeutic efficacy of 2,4-diamino-5-p-chloro-phenyl-6-ethylpyrimidine (Daraprim) in experimental toxoplasmosis. *Am. J. Trop. Med. & Hyg.*, **2**: 1037–1044.
5. Frenkel, J. K., and Hitchings, G. H., 1957. Relative reversal by vitamins (*p*-aminobenzoic, folic, and folinic acids) of the effects of sulfadiazine and pyrimethamine on *Toxoplasma*, mouse and man. *Antibiotics & Chemother.*, **7**: 630–638.
6. Eyles, D. E., 1952. *Toxoplasma* in the Norway rat. *J. Parasitol.*, **38**: 226–229.
7. Hitchings, G. H., 1955. Purine and pyrimidine antagonists. In *Symposium on the Antimetabolites. Nutrition Symposium Series*, no. 11, pp. 51–57. National Vitamin Foundation, New York.

ACTIVE IMMUNIZATION OF MAN AGAINST THE VENOM OF THE AUSTRALIAN TIGER SNAKE (*NOTECHIS SCUTATUS*)

SAUL WIENER*

Commonwealth Serum Laboratories, Melbourne

As a result of the pioneer work of Sewall in 1887,[1] it has become established that animals can be actively immunized against snake venom by the injection of increasing amounts of venom or its products. Relatively few attempts have been made since then to use a similar method for the active immunization of man.

Primitive natives of many regions where venomous snakes are found believed that protection against snake bite could be acquired by the inoculation of snake venom. The natives of Mexico introduced rattlesnake venom into various parts of the body by the use of a snake fang.[2] There is still a popular belief in our own times that people exposed to repeated bites by venomous snakes develop some degree of protection. Klauber[3] has recently reviewed the primitive beliefs and the early literature dealing with acquired and artificial immunity against snake venom in man.

Under the conditions it was generally acquired by man in ancient times, any immunity against snake venom could have been only slight and of short duration. Furthermore, the criteria available for judging the immune state of an individual were not reliable because of the variable factors which operate at the time of a snake bite and influence the amount of venom injected. Absence of symptoms may be due to either the injection of too little venom at the time of the bite or the presence of an acquired immunity. After a large amount of venom has been injected by a snake, symptoms may occur even in the presence of an acquired immunity if the extent of this immunity is not sufficient to neutralize all the venom which has been absorbed.

Whether or not an individual has acquired an active immunity against snake venom, and the extent of this immunity, can only be established with certainty by the injection of amounts of venom known to be lethal to man or by testing for circulating antivenene. The validity of conclusions which are based on the clinical picture alone can always be challenged. This may be illustrated from a recent analysis by Watt *et al.*[4] of the effects of ten poisonous bites which occurred in one patient. These authors concluded that there was no evidence from their case that immunity develops as a result of repeated bites. However, if one assesses the degree of immunity by the method used by the authors themselves, viz., by the severity of the symptoms produced, and excluding the first bite where no immunity could have existed, we find that two of the bites were severely toxic, two were moderately toxic and five produced minimal local symptoms. These results are as much in favor of a developed immunity as against it.

More recently, in an informative analysis of the effects of repeated snake-bite in 12 individuals, Parrish and Pollard[5] found no clinical or laboratory evidence that any permanent immunity had been acquired to pit-viper venom. Unfortunately, only limited data were supplied by these authors concerning the method of testing for circulating antivenene; but from the information supplied it can be inferred that protective amounts of antivenene were not present in the sera of their patients.

Under more controlled conditions another subject was immunized with increasing doses of Cape Cobra venom over a period of several years.[6] During this time he recovered, without specific treatment, after several accidental bites from species related and unrelated to the Cape Cobra. Although his antivenene titre was not determined, the fact that he eventually tolerated 40 mg of Cape Cobra venom proved that a substantial degree of active immunity had been produced.

Aims of active immunization against snake venom. In the production of active immunity against diseases caused by bacterial exotoxins such as tetanus, the aim is not only to provide a protective level of circulating antitoxin, but also to produce a state of sensitization so that a booster dose of toxoid can stimulate the rapid formation of more antitoxin. Because the amount

* Present address: Prince Henry's Hospital, Melbourne.

of bacterial toxin which is liberated in the body increases only gradually as the bacteria multiply, a booster injection of toxoid at the beginning of this incubation period will usually prevent tetanus even when the amount of antitoxin circulating at the moment of infection is insufficient to protect.

However, in the case of a snake bite, the maximum amount of venom to which the body will be exposed is injected at the time of the bite. Symptoms of envenomation may appear in less than one hour. Therefore, in the active immunization of man against snake venom, the object is to produce sufficient circulating antivenene so that all the venom introduced into the body can be neutralized. In addition, it is desirable to maintain a high level of circulating antivenene for as long as the individual requires protection against snake bite.

The active immunization against tiger snake venom which is reported here was undertaken at the request of the subject because of his sensitivity to horse serum and his hobby of keeping snakes.

METHOD AND MATERIALS

The tiger snake venom which was available consisted of the dried whole venom of the snake and also contained cellular elements and bacteria.

In order to reduce the severity of any allergic reaction which might develop when venom containing various antigenic fractions was injected repeatedly, it was considered desirable to partially purify the venom before use. It was also proposed initially to exclude the coagulant fraction from the material intended to be used for immunization. The production of an active immunity against the coagulant fraction was not considered important for protection because the lethal effects of tiger snake venom when injected subcutaneously, as is usually the case in a bite, are mainly caused by the neurotoxic component.[7, 8]

Rosenfeld and Rubinstein[9] have described the use of hydrochloric acid for the separation of the neurotoxic from the coagulant fraction of tiger snake venom. When the method used by these authors was followed, it was not found possible to free the neurotoxin from clotting activity. In several attempts at separation the neurotoxic fraction had still retained its ability to coagulate plasma. However, since acid treatment did remove certain components of venom and as the venom which remained was slightly more toxic than the original material, some degree of purification had occurred. It was therefore decided to utilize a modification of this method in order to obtain a material which, although still possessing clotting activity, was somewhat purer than crude venom and hence more suitable for injection into man.

Preparation of purified venom and toxoid. A solution of tiger snake venom was prepared by dissolving 300 mg of crude dried venom in 30 ml of distilled water. The mixture was allowed to stand at room temperature for several hours and stirred occasionally. An insoluble residue consisting mainly of cellular elements remained and was removed by centrifuging the mixture. The dry weight of this insoluble residue was 6.9 mg. To the clear supernatant 1.8 ml of concentrated hydrochloric acid was added slowly and a flocculent precipitate resulted. The mixture was allowed to stand overnight at 4°C and then centrifuged. The precipitate, which was removed, weighed after drying 14.4 mg. It had lost most of its toxicity but had retained the ability to coagulate plasma.

The supernatant was dialyzed in a cellophane bag against distilled water for 48 hours, by which time it was neutral to litmus paper. It was then freeze-dried and a white flaky material of purified venom weighing 213.3 mg was obtained.

The purified venom was used to make up solutions for use in immunization and also to prepare toxoid. It was intended to use toxoid instead of ordinary venom at some stage during the course of immunization in order to compare its antigenicity with that of unmodified venom.

Toxoid was prepared by incubating a solution containing 15 mg of venom per ml and 0.1% of neutral formalin at 37°C. At varying intervals of time thereafter, the toxicity of the venom solution was tested in mice (Table 1). After 13 days of incubation, when the toxicity of the venom solution had been reduced to $\frac{1}{20}$ of its original toxicity, the pH of the solution was increased from 6 to 7.8 by the addition of sodium carbonate in order to hasten the toxoiding process.[10] After a further period of 6 days, the toxicity of the venom was $\frac{1}{60}$ of the original value. Some of the toxoid was now removed and stored at 4°C for later use as an immunizing agent.

Preparation of venom solution for injection. The

TABLE 1

Loss of toxicity of tiger snake venom during incubation at 37°C with 0.1% formalin

Period of incubation in days	LD_{50} dose in mg*	Toxicity/original toxicity
0	0.001	1
5	0.02	1/20
13	0.02	1/20
14†	—	—
20	0.13	1/130
21‡	—	—
27	0.6	1/600
37	0.7	1/700
4 months	1.0	1/1000
9 months	1.1	1/1100

* In mice weighing 15-20 g by the intraperitoneal route.

† Sodium carbonate added to increase pH from 6 to 7.8.

‡ Portion removed for use as immunizing agent.

diluent used for making solutions of venom consisted of normal saline solution containing 1:10,000 Merthiolate. A concentrated solution of purified venom was Seitz-filtered and further amounts of diluent were passed through the filter to yield a final concentration of venom of 10 mg/ml. From this stock solution of venom, dilutions of 1:1000, 1:100 and 1:10 were prepared as required during the course of immunization. The stock solution and the dilutions made from it were stored in multiple dose containers at 4°C. Every 3 months a fresh concentrated venom solution was prepared.

Tests for sterility were carried out on all solutions under both aerobic and anaerobic conditions. In addition, the toxicity of the venom solution was assayed in mice. Before Seitz-filtration the LD_{50} dose of venom was 0.0005 mg while after filtration the LD_{50} dose was 0.001 mg. Thus, some loss of venom had occurred during the process of filtration. However, the toxicity of the Seitz-filtered concentrated venom solution showed no reduction after storage at 4°C for 3 months.

The toxoid was also Seitz-filtered, tested for sterility and dispensed in a multiple dose container in a concentration of 12 mg/ml with 1:10,000 Merthiolate added as a bacteriostatic.

Assay of antivenene. The level of circulating antivenene which was present at different periods of time during the course of immunization was determined by mixing varying amounts of the patient's serum with a constant amount of venom. The mixtures were incubated at 37°C for 1 hour and then injected by the intraperitoneal route into mice weighing 15 to 20 g. The amount of venom which was used depended on the expected level of antivenene in the serum. During the early stages of immunization when the antivenene titer was low only 3 to 6 LD_{50} doses of venom could be used while later on 10 to 15 LD_{50} of venom were added to each dilution of serum. Whenever the level of antivenene was being determined in a sample of serum, the toxicity of the venom used was assayed simultaneously. The LD_{50} dose of venom was calculated according to a method described by Reed and Muench[11] from results obtained when the following amounts of venom (in mg) were injected into each of 5 mice respectively: 0.0002, 0.0005, 0.001, 0.002, 0.003 and 0.004. Both purified and crude preparations of tiger snake venom were used in these assays.

Results were expressed in units of antivenene per ml of serum, 1 unit being the amount of serum required to neutralize 0.01 mg of venom.

The patient. The subject whom it was intended to actively immunize against tiger snake venom was a 46 year old male who was in good health and weighed 70 kg. For many years his hobby had been the study of snakes and the collection of venom from them.

In the pursuit of these activities he had been bitten several times by various snakes. From data supplied by the patient and physicians who had attended him, a record of these bites was compiled and is shown in Table 2. It is probable that additional bites occurred but have not been recorded. Allergic reactions developed in 1954 and 1955 when antivenene was injected after a bite. Although a subsequent administration of antivenene in 1957 was not followed by allergic reactions, their previous occurrence indicated that the patient had acquired sensitivity to horse serum.

During the course of immunization another bite occurred on 21st January, 1959, while the patient was handling a brown snake (*Demansia nuchalis*) from Western Australia measuring about 1 foot in length. The snake bit his right ring finger and hung on for a few seconds. The patient made no attempt to shake it off or to treat the bite as he considered the snake too small for the bite to be dangerous. He felt a slight sting during

TABLE 2

History of previous snake bites

Year	Species of snake	Treatment	Effect of bite
1945	*Demansia psammophis* (Brown Snake)	Nil	Local swelling & pruritis
1949	*Notechis scutatus* (Tiger Snake)	Antivenene	Nil
1950	*Denisonia flagellum*	Nil	Local swelling & pruritis
1951	*Demansia psammophis*	Nil	Local swelling & pruritis
1953	*Acanthopis antarticus* (Death Adder)	Antivenene	Nil
1954	*Notechis scutatus*	Antivenene	Generalized rash & pruritis
1955	*Pseudechis australis* (King Brown Snake)	Antivenene	Generalized rash
1956	*Demansia textilis*	Nil	Nil
1957	*Notechis scutatus*	Antivenene	Nil

TABLE 3

Course of immunization and antivenene titers produced

Date of injection and/or venipuncture	Amount and material injected in mg	Route of injection	Reactions	Antivenene titer units/ml
17/3/58	0.002 Venom	S.C.	Nil	0.2
25/3/58	0.004 Venom	S.C.	Nil	
1/4/58	0.008 Venom	S.C.	Nil	
15/4/58	0.02 Venom	S.C.	Nil	
22/4/58	0.05 Venom	S.C.	Local muscle stiffness	
29/4/58	0.09 Venom	S.C.	Nil	0.5
6/5/58	0.15 Venom	I.D.	Local erythema & swelling	
14/5/58	0.4 Venom	I.D.	Local wheal & pruritis	
20/5/58	0.8 Venom	S.C.	Local muscle pain	
27/5/58	1.3 Venom	S.C.	Local ache & stiffness	
10/6/58	2.4 Venom	S.C.	Nil	1.0*
24/6/58	3.2 Venom	S.C.	Nil	
1/7/58	6.0 Venom	S.C.	Nil	3.2
8/7/58	6.0 Toxoid	S.C.	Local muscle stiffness	5.1
15/7/58	12.0 Toxoid	S.C.	Local swelling	
29/7/58	24.0 Toxoid	S.C.	Nil	3.0
5/8/58	— —	—	—	3.0
11/8/58	2.7 Venom	S.C.	Local swelling, muscle pain & stiffness; slight pyrexia	
2/9/58	3.6 Venom†	S.C.	Slight local stiffness	3.4
4/9/58	9.0 Venom†	S.C.	Swelling & mild pyrexia	
17/9/58	— —	—	—	4.5
21/10/58	— —	—	—	3.0
13/11/58	36.0 Toxoid	S.C.	Joint pains during night	2.5
16/12/58	— —	—	—	2.0
23/12/58	— —	—	—	1.5
29/1/59	— —	—	—	4.5
17/3/59	3.6 Venom	S.C.	Local swelling	4.0
25/3/59	9.0 Venom	S.C.	Swelling of whole arm	4.0
8/4/59	15.0 Venom†	I.M.	Local swelling	3.3
15/4/59	25.0 Venom	I.M.	Local muscle stiffness with tenderness	3.5
21/4/59	— —	—	—	4.2
13/5/59	— —	—	—	4.4
4/6/59	— —	—	—	5.2
2/7/59	— —	—	—	2.5
4/8/59	— —	—	—	2.0

* Precipitins detectable in serum.

† 50–75 mg Antistine® injected.

the bite and squeezed some blood-stained fluid from the two fang marks.

The bite was forgotten and not remembered when headache and some tightness in the chest developed 30 minutes later. However, 1 hour after the bite, marked dyspnea caused the patient to sit down and to realize that his symptoms were due to the bite. He then collapsed and remained unconscious for 15 minutes. This was followed by profuse perspiration and blurred vision.

When seen 2 hours after the bite he was pale, felt weak and complained of nausea and headache. Dyspnea was no longer present. There was an enlarged tender lymph gland in the right axilla. The blood pressure was 140/90 and the pulse was of normal volume with a rate of 59 per minute.

No antivenene was given but 100 mg of Antistine® was injected in case antivenene was required later on. The patient was hospitalized and 4 hours after the bite all symptoms had disappeared, except for a feeling of weakness which persisted for 2 days. The next day there was some swelling and itchiness at the site of the bite which was also tender.

Course of immunization. The course of immunization extended over 13 months during which period 24 injections were given (Table 3). At the beginning of the course injections were given at intervals of approximately 1 week, but thereafter, intervals up to 4 months elapsed between injections. The initial dose of venom was 0.002 mg and this was gradually increased until 25 mg was administered at the final injection. These doses have been calculated from the amount of venom used to make up the solution before Seitz-filtration.

Usually injections were given subcutaneously into the upper arm but the intradermal and intramuscular routes were each used on 2 occasions. On 4 occasions toxoid was injected instead of venom.

Because of the local reactions which occurred, a single dose of 50–75 mg of Antistine was injected with the venom on several occasions.

At various periods of time during the course of immunization white cell counts were performed and the urine was examined for abnormal constituents. In addition, samples of blood were taken for determining the level of circulating antivenene.

RESULTS

Level of circulating antivenene. Antivenene was detectable in the serum of the patient before immunization with venom had been commenced. From the results of the assay, which are shown in Table 4, it can be estimated that the preimmunization antivenene titer was approximately 0.2 units/ml. Only 3 LD_{50} doses of venom could be used in testing for the low level of antivenene which was present. The specific nature of the observed neutralization was confirmed by using, as a control, the serum of a person who had never been bitten by a snake. The control serum alone was not toxic when 1 ml was injected into each of 3 mice by the intraperitoneal route. However, when 2 LD_{50} doses of venom were added to 1 ml of this serum all the injected animals died.

The levels of circulating antivenene which were produced as a result of active immunization are shown in Table 3. The antivenene titer gradually rose during the first few months of immunization. By the end of the 4th month the titer was 5.1 units/ml 1 week after an injection of 6 mg of venom had been given (Table 5). Precipitins were also detectable in the serum when first tested for at the time when the antivenene titer was 1 unit/ml. Further injections of increasing amounts of venom or toxoid during the next 9 months did not result in any appreciable increase in the titer above 5 units/ml. The injection of toxoid was even followed by a fall in the antivenene titer which persisted until further injections of venom were given.

A rise in the antivenene titer occurred after the patient was bitten by a brown snake (*D.*

TABLE 4

Neutralization of tiger snake venom by serum of patient prior to immunization

Amount of serum in ml	Amount of venom* in mg	No. of mice	No. that died
0.3	0.003	3	3
0.5	0.003	3	3
0.7	0.003	3	3
0.9	0.003	3	0
0.9	0.005	3	3
1.0†	0.002	3	3

* LD_{50} dose of venom = 0.001 mg.

† From another subject who has never been exposed to a snake bite.

TABLE 5

Neutralization of tiger snake venom by serum of patient one week after an injection of 6 mg of tiger snake venom

Amount of serum in ml	Amount of venom* in mg	No. of mice	No. that died
0.24	0.02	3	3
0.28	0.02	3	3
0.32	0.02	3	3
0.36	0.02	3	1
0.40	0.02	3	0
0.44	0.02	3	0

* LD_{50} dose of venom = 0.0015 mg.

nuchalis). A previous determination of the titer which had been made about 5 weeks before the bite showed that 1.5 units/ml were present. Eight days after the bite the titer had risen to 4.5 units/ml. No injections of venom had been given between these 2 periods.

There was a gradual fall in the level of circulating antivenene when periods up to 4 months elapsed between injections during the last 7 months of immunization. The antivenene titer at different times during this period fluctuated between 1.5 and 4.5 units/ml. Seven weeks after the last dose of 25 mg of venom, the antivenene titer was 5.2 units/ml. No further injections were given and 4 months after the last dose the antivenene titer was 2 units/ml.

Reactions. Throughout the whole period of immunization during which a total of approximately 82 mg of tiger snake venom and 78 mg of toxoid were administered, the general health of the patient was not adversely affected. His appetite remained good and there was no loss of weight. At no stage were any abnormal constituents detected in the urine; there was no eosinophilia and the white cell count ranged from 6,300–12,000/cmm.

No reactions occurred after the first 4 injections of venom. However, many of the subsequent injections were followed by local reactions which consisted mainly of varying degrees of swelling at the site of the injection. Local muscle stiffness, pain and tenderness were also present occasionally (Table 3). Malaise with slight pyrexia and generalized joint and muscle pains occurred on 3 occasions and lasted for 24 hours.

Local reactions occurred when venom was injected by both the subcutaneous and intramuscular routes. On the 2 occasions when venom was injected intradermally early in the course of immunizations, an immediate wheal with pseudopodia and erythema resulted. During the next few hours the wheal continued to enlarge, became tender and there was pruritis of the axilla of the injected side. A similar immediate wheal formed when venom was injected intradermally about 3 months after the last immunizing injection of venom.

When a local reaction did occur, its severity did not appear to be related to the amount of venom injected nor to the interval which had elapsed since the previous injection. It also occurred when toxoid was used. As the occurrence and intensity of local reactions after the injection of either venom or toxoid were not predictable, it was difficult to determine whether the simultaneous administration of an antihistamine exerted any beneficial effect. The injection of 50 to 75 mg of Antistine® did not prevent the occurrence of local reactions.

Generally, whenever a local reaction occurred it had disappeared 24 to 48 hours after the injection. Occasionally some tenderness persisted at the site of injection for 1 or 2 more days. Beyond some limitation of elbow movement when the swelling involved the elbow region on 1 occasion, the patient was not incapacitated and continued in his usual occupation on the day of injection.

DISCUSSION

It is not known what constitutes a lethal dose of tiger snake venom for an adult man. On the basis of results obtained when known amounts of venom were injected into larger animals such as the sheep, goat and horse,[12] it is probable that the lethal dose for a man weighing 70 kg is in the range of 0.35 to 1.4 mg of venom. Since our subject was able to tolerate 25 mg of venom, he must have acquired a substantial degree of immunity.

The minimum extent of this acquired immunity can be estimated from the level of the circulating antivenene which was present. At the height of his immune response, our subject had about 5 units of antivenene per ml of serum. As the circulating plasma volume constitutes 5% of the body weight, 17,500 units of antivenene were circulating in his blood at this stage. Such an amount of antivenene is sufficient to neutralize 175 mg of venom. Even with an antivenene titer of 1 unit per ml which was reached relatively

early in the course of immunization, our subject would have tolerated at least 35 mg of venom.

The actual degree of protection conferred as a result of active immunization is higher than appears from an estimate based merely on the total amount of antivenene which is present in the circulation at any time. Such circulating antivenene constitutes only part of the total antivenene present in the body since about 50% of the total γ-globulin in a normal person is found extravascularly.[13] The presence of antivenene in tissue spaces has been demonstrated in the case of animals immunized against cobra venom.[14]

In clinical practice the aim in the treatment of a bite by a venomous snake is to introduce with as little delay as possible after the bite, sufficient specific antivenene into the circulation of the patient to neutralize the venom which the snake has injected. The recommended procedure in the case of a bite by a tiger snake is to give an initial dose of 3,000 units by the intravenous route.[15] This amount of antivenene will neutralize in vitro 30 mg of venom which is approximately the average venom yield of a tiger snake.[16] However, if there has been any delay in treatment or if the early onset of severe symptoms suggests that more than the average amount of venom has been injected—and individual tiger snakes have been known to yield up to 189 mg of venom—then considerably higher doses of antivenene have to be given as determined by the response of the patient. In order to neutralize the toxic effects of venom in vivo, the amount of antivenene required has to be increased as the time interval between envenomation and antivenene administration increases. Presumably for an appreciable period of time during envenomation the attachment of neurotoxin to susceptible cells can be reversed by an excess of antivenene.

In an actively immunized person, venom introduced as a result of a bite is neutralized to some extent before it enters the blood stream or lymphatics, while after absorption it is neutralized by antivenene which is already present in the circulation. The sustained presence of only 1 unit of antivenene per ml of serum in an actively immunized subject provides a degree of protection which an injection of 3,000 units of heterologous antivenene after the bite cannot match.

The presence of circulating antivenene in the serum of the patient before immunization had been started provided hope that during active immunization our subject would produce a higher antivenene titer than was actually the case. There can be little doubt that the antigenic stimulus responsible for the presence of antivenene at this stage was supplied by the venom which tiger snakes and other snakes had injected when our subject was bitten on at least 9 previous occasions. Although Parrish and Pollard[5] were unable to detect circulating antivenene in 12 subjects who had been repeatedly bitten by various snakes, they could demonstrate, in the serum of some of their subjects, the presence of reagins which passively sensitized the skin of normal people to pit-viper venom. Only three of their subjects had experienced as many or more bites than did our patient. In any case, the low preimmunization titer of about 0.2 unit/ml which was present in our patient cannot be considered to constitute sufficient protection against an average tiger snake bite.

The reason for the rise in titer which followed the bite by *Demansia nuchalis* during the course of immunization is a matter for speculation. Monovalent tiger snake antivenene exerts little cross-protection against the venom of the common brown snake (*Demansia textilis*). That the same also applies to the venom of *D. nuchalis* is evident from the fact that our subject suffered intoxication from the small amount of venom injected by this snake at a time when he had appreciable amounts of tiger snake antivenene in his circulation. The most likely explanation for the observed rise in titer is that the venom of *D. nuchalis* contains, in addition to its own toxic antigens, a highly active antigen which is also present in tiger snake venom. Confirmation of this theory must await the production of an antivenene against the venom of *D. nuchalis*.

The reactions which occurred during the course of immunization were less severe than anticipated. H. E. Haast reported to the author that he noticed slight toxic effects on several occasions during his immunization with cobra venom in 1957. Since the reactions in our patient occurred after the administration of both venom and toxoid, it is unlikely that a direct toxic effect of venom was responsible. The patient also stated that the reactions were unlike any which he had experienced when bitten by venomous snakes on previous occasions. Asthma and hay-fever have been known to follow the prolonged handling of snakes or repeated exposure to snake

venom. Cutaneous sensitivity to snake venom was also demonstrable after repeated snake bite,[5] and in one case generalized urticaria occurred after a bite.[17] However, the repeated injection of increasing amounts of venom was used to desensitize individuals who had been sensitive to venom.[18] The presence of precipitating antibodies in the serum of our patient suggests that the local reactions which occurred were due to the combination of antigen with antibody at the site of injection. The occurrence of an immediate wheal when venom was injected intradermally supports such a view.

The localization and partial neutralization of venom at the site of injection may also have been partially responsible for the failure of relatively large doses of venom to raise the antivenene titer above about 5 units per ml. It is possible that with still larger doses of venom than were used, a higher titer of antivenene might have been obtained. However, it must be remembered that individuals vary greatly in their ability to produce antibodies. When horses are being hyperimmunized with snake venom, a stage is usually reached when further injections of venom produce no appreciable increase in the level of circulating antivenene.

Before a solution of snake venom can be injected safely into man, a sterile preparation of known potency must be available. Bacteria are frequently present both in dried venom and in freshly collected liquid venom.[19] Severe and occasionally fatal results have followed the use of venom contaminated by bacteria.[20]

The active immunization of man against the venom of the tiger snake is a practical and safe procedure provided sterile solutions of known toxicity are used. From the results obtained in this investigation, it appears unnecessary to increase the dose of venom, during the primary course of immunization, beyond 6 mg. An appreciable degree of protection will be produced and persist for several months. It can be maintained by the injection of further stimulating doses of venom every 3 months. Further work is required to establish whether man can be immunized against the venom of other Australian snakes.

The indications for active immunization against snake venom are those which led to the immunization of our subject, viz., sensitivity to horse serum in an individual who is constantly handling venomous snakes. Individuals who handle snakes, even if they are not sensitive to horse serum, and those who are going to isolated areas where snakes occur and no medical attention is available, may also be suitable subjects for active immunization. Since many injections are necessary to induce and maintain an effective immunity against snake venom, active immunization is not justified, nor likely to be acceptable, in other cases. Toxoid had no demonstrable antigenic effect and cannot be used to replace venom. Otherwise it might have been possible to reduce the number of doses required. Such a reduction may become feasible if toxoid can be prepared by a method which does not impair its antigenicity and by the use of a suitable adjuvant with which to prolong the antigenic stimulus.

SUMMARY

A method is described for actively immunizing man against the venom of the tiger snake.

Over a period of 13 months, increasing amounts of venom were injected into a subject who had been repeatedly bitten by venomous snakes, and who was sensitive to horse-serum. The initial dose was 0.002 mg and the final dose 25 mg of venom.

A pre-immunization titer of 0.2 units of antivenene per ml of serum was present. Three months after the commencement of immunization the titer was 1 unit/ml, while at 4 months a titer of 5.1 units/ml was reached. Depending on the interval of time which then elapsed between injections, the antivenene titer fluctuated between 1.5 and 5.2 units/ml. Four months after the final dose a titer of 2 units/ml was still present.

Venom treated with formalin (toxoid) did not exert a detectable antigenic effect.

The health of the patient was not adversely affected throughout the course of immunization, but transitory local reactions frequently followed the injections of either venom or toxoid. On three occasions mild general reactions occurred.

Evidence is supplied which suggests that after active immunization a titer of 1 unit/ml constitutes in an adult a protective level of antivenene against the bite of a tiger snake containing an average amount of venom.

The nature of the local reactions, the protec-

tion afforded by active immunization and the indications for active immunization are discussed.

ACKNOWLEDGMENT

The help and co-operation of the subject of this study, Mr. C. Tanner, is gratefully acknowledged.

REFERENCES

1. Sewall, H., 1887. Experiments on the preventive inoculation of rattlesnake venom. *J. Physiol.*, **8:** 203–210.
2. Calmette, A., 1908. *Venoms, Venomous Animals and Antivenomous Serum-Therapeutics.* London.
3. Klauber, L. M., 1956. *Rattlesnakes; Their Habits, Life Histories and Influence on Mankind*, vol. 2. University of California Press, Berkeley.
4. Watt, H. F., Parrish, H. M., and Pollard, C. B., 1956. Repeated poisonous snakebites in the same patient. *North Carolina M. J.*, **17:** 174–179.
5. Parrish, H. M., and Pollard, C. B., 1959. Effects of repeated poisonous snakebites in man. *Am. J. M. Sc.*, **237:** 277–286.
6. Haast, W. E., and Winer, M. L., 1955. Complete and spontaneous recovery from the bite of a Blue Krait Snake (*Bungarus caeruleus*). *Am. J. Trop. Med. & Hyg.*, **4:** 1135–1137.
7. Tidswell, F., 1906. *Researches on Australian Venoms.* Sydney.
8. Kellaway, C. H., 1938. The symptomatology and treatment of the bites of Australian snakes. *M. J. Australia*, **2:** 585–589.
9. Rosenfeld, S., and Rubinstein, J., 1941. Separation of the coagulant from the toxic principles of the venom of the Australian tiger-snake (*Notechis scutatus*). *J. Lab. & Clin. Med.*, **27:** 45–49.
10. Boquet, P., and Venderly, R., 1943. Influence de pH sur la transformation du venin de cobra en anavenin par l'aldehyde de formique. Preparation d'un anavenin solide. *Compt. Rend. Soc. biol.*, **137:** 179–180.
11. Reed, L. J., and Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, **27:** 493–497.
12. Kellaway, C. H., 1929. The venom of *Notechis scutatus*. *M. J. Australia*, **1:** 348–358.
13. Gitlin, D., 1957. Some concepts of plasma protein metabolism. *Pediatrics*, **19:** 657–671.
14. Kellaway, C. H., and Williams, F. E., 1932. Some observations on cellular immunity to snake venom. *J. Path. & Bact.*, **35:** 193–197.
15. Commonwealth Serum Laboratories, 1956. *Handbook of Instructions*, pp. 62–71. Melbourne.
16. Freeman, Mavis, 1934. The venom yields of common Australian poisonous snakes in captivity. *M. J. Australia*, **2:** 373–377.
17. Lounsberry, C. R., 1934. Rattlesnake anaphylaxis associated with a generalized dermatitis. *Arch. Dermat. & Syph.*, **29:** 658–667.
18. Peck, S. M., 1933. Sensitization and desensitization in man with snake venom (*Ancistrodon piscivorus*). *Arch. Dermat. & Syph.*, **27:** 312–315.
19. Williams, F. E., Freeman, M., and Kennedy, E., 1934. The bacterial flora of the mouths of Australian venomous snakes in captivity. *M. J. Australia*, **2:** 190–193.
20. Anderson, J. F., 1914. Danger in the subcutaneous injections of solutions of crotalin. *J. A. M. A.*, **62:** 893–895.

THE EFFECT OF ANTI-ERYTHROCYTIC ANTIBODIES UPON *PLASMODIUM BERGHEI* INFECTIONS IN WHITE MICE*

THOMAS M. SCHWINK†

Rutgers University, College of South Jersey, Camden, New Jersey

Chickens receiving injections of anti-erythrocytic antibodies experience lower *Plasmodium lophurae* parasitemias than do control chickens.[1] The action of these antibodies is not merely the result of their aiding in the removal of the infecting inoculum of parasitized erythrocytes, for lower parasitemias were obtained even when administration of these antibodies was begun 2 days after infection.

The present study was undertaken to determine whether such antibodies would similarly affect *P. berghei* infections in mice. If the antimalarial effect of anti-erythrocytic antibodies is a universal phenomenon applicable to malaria infections in general, similar results should be obtained with *P. berghei* and *P. lophurae*.

These two species of malaria parasites have different erythrocyte preferences. *P. berghei* prefers immature erythrocytes, while *P. lophurae* prefers mature erythrocytes. The results obtained in the present study were different from those obtained with *P. lophurae*, and this difference is likely attributable to the different erythrocyte preferences of the parasites.

MATERIALS AND METHODS

The Kasapa strain of *P. berghei* used in these investigations was kindly furnished by Dr. G. Robert Coatney. It was maintained by serial transmission through White Swiss mice. White Swiss mice served as hosts in all the series. They were infected when 41 to 86 days old by inoculation with parasitized mouse blood. In the first of six series the parasitized blood was administered intraperitoneally, while in the other series infection was via the intravenous (tail vein) route.

Serum was obtained from white rabbits. Immune serum was obtained from rabbits which had received 4 to 6 intravenous and intramuscular injections of saline-washed normal mouse erythrocytes. Normal serum was from a rabbit which had never received mouse erythrocytes. Sera were stored frozen without the addition of any preservative.

In each series the first serum injection given each mouse was via the intravenous route. Subsequent injections were administered intraperitoneally. Day 0 injections were given about 2 hours prior to infection. Quantities of parasitized cells and serum were scaled according to the weight of the recipient. They are expressed as quantities per 100 g of body weight. The number of parasitized cells administered was 5×10^6 per 100 g in all series. This quantity was only approximated except in the first series, where it was accurately measured. However, in each series all of the mice received the same quantity per unit of body weight.

Thin blood smears were made daily or on alternate days starting on Day 3 and continuing until the inevitable death of the mice. The smears were fixed in methyl alcohol, dried, and stained with Giemsa stain. Percentages of mature and immature erythrocytes, and percentages of each of these with parasites, were recorded. The counting procedure and the method of determining statistical significance were the same as used previously.[1]

RESULTS

Percentages of parasitized erythrocytes for the first five series are shown in Table 1. In each of these experiments mice receiving immune rabbit serum experienced severe infections earlier than did control mice. The response was the same when serum administration was begun on Day 2 as when it was begun on Day 0.

In the 3rd and 5th series, immune serum recipients showed significantly lower parasite counts early in the course of the infection (3, or 4 and 5 days after inoculation) than did control mice. In Series 3, Day 3, parasitemias of Groups 1 and 3 differed significantly at the 5% level of probability. In Series 5 the two groups differed

* This study was supported by a grant from the Rutgers University Research Council.

† Present address: Division of Veterinary Science, College of Agriculture, University of Wyoming, Laramie, Wyoming.

significantly at the 5% level of probability on Day 4 and at the 1% level on Day 5. In both experiments the serum recipients showing this response received relatively small (0.3 ml) injections of immune serum. This response was transient and these mice later showed higher parasite counts than did the control mice.

Series 6, not shown in Table 1, consisted of three groups of seven mice each. Serum and saline dosages were 0.2 ml on days 0–7. Group 1

TABLE 1

Percentages of parasitized erythrocytes of surviving mice (Seven mice per group in each series. M is the group mean and SD the standard deviation. Serum was from rabbits immunized against normal mouse erythrocytes. Day 0 is the day of infection.)

Series	Group and treatment	Data	% of erythrocytes parasitized at						
			3 days	4 days	5 days	6 days	8 days	11 days	16 days
1	1 saline*	M	1.0	6.7	10.0	10.9	10.3	14.3	21.1
		SD±	0.5	5.8	3.9	3.6	4.4	5.7	4.2
	2 serum*	M	1.3	28.0	37.0	25.3	60.3	¶	
		SD±	1.0	20.7	28.2	14.5	11.0		
2	1 saline†	M	2.9	6.1	4.2	2.7	5.1	6.0	19.9
		SD±	1.5	3.1	2.1	1.1	3.4	4.2	10.3
	2 serum†	M	4.5	4.6	3.8	4.6	36.1	16.9	21.8
		SD±	4.3	1.3	1.3	1.5	19.0	6.3	2.5
3	1 none	M	1.0	6.0	4.9	4.0	6.9	19.5	33.8
		SD±	0.4	1.4	1.9	2.2	3.4	10.9	7.8
	2 serum*	M	1.0	5.9	15.2	12.5	22.4	40.1	¶
		SD±	0.7	4.3	17.5	1.9	6.3	21.5	
	3 serum‡	M	0.5	3.9	7.1	7.1	18.6	31.5	28.8
		SD±	0.4	4.2	6.9	2.7	8.8	15.4	
4	1 serum§	M	4.1	5.1	9.4	18.1	37.3	24.8	43.5
		SD±	1.3	1.8	3.7	12.8	12.2	9.1	
	2 serum†	M	4.2	5.1	7.2	18.2	40.5	33.1	24.5
		SD±	1.6	1.9	3.4	10.8	13.6	4.7	
	3 none	M	3.8	6.9	7.5	6.8	7.3	10.4	23.5
		SD±	1.5	1.8	2.0	3.2	1.7	5.3	5.0
5	1 saline‖	M	1.6	8.4	6.9	4.2	7.3	6.9	16.2
		SD±	0.6	4.2	2.6	2.2	3.6	2.7	10.1
	2 serum‖	M	1.5	4.3	3.5	5.8	30.7	31.3	33.2
		SD±	0.6	1.9	1.1	4.1	11.3	10.4	9.1

* 0.6 ml on days 0–2.
† 0.6 ml on days 2–4; 0.4 ml on day 5.
‡ 0.3 ml on days 0–2.
§ 0.6 ml on days 0–2; 0.4 ml on day 3.
‖ 0.3 ml on days 0–7.
¶ All mice dead.

TABLE 2

Survival times and percentages of immature erythrocytes

Series	Group	Data	Day of death	Percentages of immature erythrocytes at						
				3 days	4 days	5 days	6 days	8 days	11 days	16 days
1	1	M	15.4	5.2	3.9	2.0	1.5	4.7	9.3	15.8
		SD±	5.1	2.8	2.8	1.3	1.0	2.2	4.1	2.0
	2	M	7.0	23.5	48.2	53.8	24.7	35.0		
		SD±	2.8	18.7	11.7	11.6	5.2	21.4		
2	1	M	21.6	0.5	1.0	0.9	1.2	4.2	6.6	18.5
		SD±	4.9	0.7	0.7	0.7	0.6	3.1	4.0	9.5
	2	M	14.6	1.9	1.4	3.4	4.3	35.2	16.0	20.3
		SD±	4.6	1.4	0.8	1.7	1.7	19.8	7.2	0.3
5	1	M	19.7	1.6	1.0	0.8	1.4	5.7	6.5	15.5
		SD±	4.2	0.8	1.0	0.8	0.8	3.8	2.6	10.0
	2	M	13.7	2.3	1.7	2.2	5.6	30.7	30.8	33.3
		SD±	4.1	0.8	1.1	1.5	5.1	12.2	11.5	7.3

received immune rabbit serum, Group 2 received normal rabbit serum, and Group 3 received saline. Results with immune serum were similar to those observed in all the other series, i.e., immune serum recipients experienced high parasitemias earlier than did controls. Normal serum recipients experienced infections similar to those of the controls. The normal serum did not alter the course of the infection. Thus 10 days after infection, with all mice still alive, parasite counts averaged 24.8 ± 7.0% for Group 1, 7.1 ± 2.0% for Group 2, and 5.9 ± 2.9% for Group 3.

Survival times and percentages of immature erythrocytes for three of the experiments are shown in Table 2. Data for the other three experiments are similar to those shown. Although all infected mice eventually died as a result of the infection, death occurred earlier among immune serum recipients than among control mice.

Immune serum recipients had higher percentages of immature erythrocytes than did control mice. This was a result of erythrocyte destruction by the serum, forcing the mice to replace many of the destroyed erythrocytes with cells that were not fully mature. Comparison of Table 1 and Table 2 shows that a relation exists between percentage of all erythrocytes parasitized (Table 1) and percentage of immature erythrocytes present (Table 2). Where there was a high percentage of immature erythrocytes there was also a high percentage of total erythrocytes parasitized. The only exception to this occurred early in Series 1 when there were not yet enough parasites to parasitize a large percentage of the high number of immature erythrocytes present.

The immature erythrocytes were heavily parasitized, while a much smaller percentage of the mature erythrocytes contained parasites. The higher percentages of total erythrocytes parasitized in immune serum recipients (Table 1) occurred because the immune serum recipients contained higher percentages of heavily parasitized immature erythrocytes.

DISCUSSION

The high parasite counts of the mice receiving immune rabbit serum resulted from the higher percentages of immature erythrocytes found in the blood of these mice. Immature erythrocytes are much preferred by *P. berghei*. In the work reported here the percentage of immature erythrocytes containing parasites was always much higher than the percentage of mature erythrocytes containing parasites. This preference of *P. berghei* for immature erythrocytes has been reported many times by previous workers (10 references listed by Zuckerman[2]).

Similarly, bleeding or the administration of hemolytic chemicals (phenylhydrazine hydrochloride or acetyl phenylhydrazine) results in higher *P. berghei* parasitemias than those experi-

enced by control mice or rats.[2, 3, 4] In all probability, the *P. berghei*-aiding actions of these agents and of anti-erythrocytic antibodies are identical and the results reported here supplement the results of previous workers on the effect of anti-erythrocytic substances upon infections with this species of malaria parasite.

P. lophurae, on the other hand, prefers mature erythrocytes.[5-8] With this species one might expect anti-erythrocytic chemicals or antibodies to reduce the parasitemia. This is just what has been reported with phenylhydrazine hydrochloride[6, 8] and with anti-erythrocytic antibodies.[1, 9]

Other investigators have reported anti-erythrocytic chemicals to decrease parasitemias with *P. knowlesi*[10, 11] and with *P. vivax*;[12] and to increase parasitemias with *P. cathemerium*.[13, 14]

Only during the early stages of the infection in Series 3 and 5 were the results obtained with *P. berghei* similar to those obtained previously with *P. lophurae*. Perhaps the antimalarial action of anti-erythrocytic antibodies is a universal phenomenon but is masked with species preferring immature erythrocytes by the action of the antibodies in stimulating the production of so many very desirable (to the parasites) immature erythrocytes. The lower parasitemias of immune serum recipients in Series 3 and 5 occurred before the percentages of immature erythrocytes in the blood of these mice reached levels much higher than those in the controls.

SUMMARY

Serum from rabbits immunized against normal mouse erythrocytes was administered to mice infected with *Plasmodium berghei* in six experiments involving a total of 105 mice. These mice were given three to eight daily serum injections, starting shortly before infection or 2 days after infection. Mice receiving this serum experienced severe infections and death earlier than did infected control mice. The blood of immune serum recipients contained high percentages of immature erythrocytes, most of which were parasitized. Normal rabbit serum had no effect upon the course of the infection in the one experiment in which such serum was used.

In two of the six experiments, immune serum recipients showed lower parasitemias than did control mice early in the course of the infection. This response was very transient and these mice later showed higher parasite counts than did the control mice.

REFERENCES

1. Schwink, T. M., 1954. Factors affecting passive immunity in blood-induced lophurae malaria of the chicken. *Am. J. Trop. Med. & Hyg.*, **3**: 232–249.
2. Zuckerman, A., 1957. Blood loss and replacement in plasmodial infections. I. *Plasmodium berghei* in untreated rats of varying age and in adult rats with erythropoietic mechanisms manipulated before inoculation. *J. Infect. Dis.*, **100**: 172–206.
3. Fabiani, G., Clausse, J., and Fulchiron, G., 1952. Intérêt de la reticulocytose sanguine dans le paludisme expérimental du Rat blanc à *Plasmodium berghei*. *Compt. rend. Acad. Sc.*, **235**: 274–276.
4. Singer, I., 1954. The effect of splenectomy or phenylhydrazine on infections with *Plasmodium berghei* in the white mouse. *J. Infect. Dis.*, **94**: 159–163.
5. Wolfson, F., 1940. Successful cultivation of avian plasmodia in duck embryos. *Am. J. Hyg.*, sect. C, **32**: 60–61.
6. Terzian, L. A., 1941. Studies on *Plasmodium lophurae*, a malarial parasite in fowls. II. Pathology and the effects of experimental conditions. *Am. J. Hyg.*, sect. C, **33**: 33–53.
7. Rigdon, R. H., and McCain, B. E., 1947. Some factors that influence the degree of parasitemia in ducks infected with *P. lophurae*. *Am. J. Trop. Med.*, **27**: 673–681.
8. Rigdon, R. H., Micks, D. W., and Breslin, D., 1953. Effect of phenylhydrazine hydrochloride on *Plasmodium lophurae* infection in the duck. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **47**: 70–76.
9. Coffin, G. S., 1951. Passive immunization of birds against malaria. *J. Infect. Dis.*, **89**: 8–15.
10. Cruz, W. O., 1949. Ação da acetilfenilhidrazina na infecção do *Macaco rhesus* pelo "*Plasmodium knowlesi*." *Rev. brasil. biol.*, **9**: 79–85. (*Trop. Dis. Bull.*, **47**: 717.)
11. Rigdon, R. H., Micks, D. W., and Breslin, D., 1950. Effect of phenylhydrazine hydrochloride on *Plasmodium knowlesi* infection in the monkey. *Am. J. Hyg.*, **52**: 308–322.
12. Lorant, S., 1946. The non-specific chemotherapy of malaria. *J. Trop. Med. & Hyg.*, **49**: 63–67.
13. Hegner, R., and Hewitt, R., 1938. The influence of young red cells on infections of *Plasmodium cathemerium* in birds. *Am. J. Hyg.*, **27**: 417–436.
14. Hewitt, R., 1939. Experimental erythroblastosis in canaries and its effect on infections with *Plasmodium cathemerium*. *Am. J. Hyg.*, sect. C, **29**: 135–148.

A NOTE ON PSYCHOSES AND LOEFFLER'S SYNDROME COMPLICATING CREEPING ERUPTION

MORRIS W. DEXTER,* HELEN L. T. DEXTER AND ALFRED H. LAWTON*

Skin eruption due to nematode larvae is a well-known problem in Florida. The prolonged pruritis with its continuous, unmitigated itching leading to loss of sleep and profound fatigue seems to present sufficient difficulties for patient and physician. However, actual psychotic episodes superimposed upon or resulting from the "creeping eruption" may further compound the situation.

During one "season of peak occurrence" of creeping eruption, 15 cases were specially observed by us. Of these, nine of the patients had very extensive skin involvement of recent origin. Three of this latter group developed actual psychotic episodes while under our care, although all 15 had been classified as mentally normal when first seen.

A brief review of the three cases of persons who manifested psychoses follows:

Case 1. A 37-year old white male was admitted to the hospital on the 5th day of evident larval migration in the skin. Response to carbon dioxide therapy was slow, and on the 13th day of his hospitalization, he attempted suicide by slashing his wrists. He was transferred to the psychiatric division and given three electric shock treatments without noticeable improvement in his mental condition. He expressed regret that he had not succeeded in his attempted suicide and stated he would try again when the opportunity arose. However, while he was in the psychiatric ward, carbon dioxide therapy of his skin lesions was continued, with gradual response. As his dermatological condition improved, his mental state underwent a corresponding and almost parallel amelioration. At the end of 8 weeks he was discharged from the hospital with his creeping eruption cured and his psychiatric status normal.

Case 2. A 49-year old white male who was hospitalized on the 2nd day of his disease was mentally normal on admission. During the 2nd week of his disease, he developed an agitated depressive state, characterized by frequent crying spells, nausea, vomiting, incessant activity and incoherent speech. This period was followed in 10 days by one of sullenness, delusion and ideas of reference. Both of these episodes were accompanied by severe itching and loss of sleep. Satisfactory response to sedation was continued, with gradual improvement in the condition. By the end of the 4th week following the onset of the psychotic interludes, the skin almost cleared, pruritis ceased and the patient's mental status simultaneously returned to normal without further psychiatric intervention.

Case 3. A 33-year old white male was admitted with extensive larval infestation of 4 days' duration. It was stated that he was well oriented and cooperative at that time. His skin lesions were treated with ethyl chloride and carbon dioxide snow, with slow response. The patient gradually became sullen and, because of pruritis, had little sleep. Two weeks after admission he developed a depressive state with poor contact. He refused medication and most food. Treatment of his skin lesions was continued, with improvement both in those and in his mental state. However, 1 week later he signed out against medical advice, with his skin not entirely clear. Since he did not return to the hospital, it is assumed that both his skin and his mental condition continued to improve.

COMMENT

It is pertinent to note that in all cases white blood counts were normal on admission but that in the three reported here leukocytosis with marked eosinophilia developed at the time of onset of the psychotic episodes. These findings returned to normal as the mental condition improved.

Similarly, chests, previously normal by x-ray, showed roentgen evidence of pneumonitis during the active phase of the creeping eruption. This, plus the eosinophilia, is suggestive of Loeffler's syndrome in association with creeping eruption, as reported by Wright and Gold.[1] It is remarkable that our patients had no symptoms or other signs referable to the pulmonary pathology in contrast with the cases reported by Muhleisen[2] in which

* Veterans Administration Center, Bay Pines, Florida.

Ancylostoma larvae were demonstrated in the sputum.

The cause of the psychotic episodes in the three cases briefly reviewed is unknown. Other dermatoses accompanied by severe and prolonged pruritis have occasionally precipitated similar episodes. Severe itching and extreme fatigue may be sufficient to trigger the psychotic state. However, we cannot avoid speculating that the pulmonary pathology, eosinophilia and the psychoses may have common etiology and give evidence of systemic intoxication. The inflammatory dermal response of the skin about the larval burrows may certainly be considered evidence of local allergic and toxic reactions.

SUMMARY

Three cases of persons who had "creeping eruption" or "cutaneous larva migrans" and developed psychoses manifested as severe mental depressions and findings suggestive of Loeffler's syndrome are presented. It is hoped this note will stimulate further reports and detailed studies to clarify the relationships observed.

REFERENCES

1. Wright, D., and Gold, E., 1945. Loeffler's syndrome associated with creeping eruption. *J. A. M. A.*, **128:** 1082–1083.
2. Muhleisen, J. P., 1953. Demonstration of pulmonary migration of the causative organism of creeping eruption. *Ann. Int. Med.*, **38:** 595–600.

SEROLOGIC DIAGNOSIS OF *SCHISTOSOMA MANSONI* INFECTIONS

I. Development of a Cercarial Antigen Slide Flocculation Test

ROBERT I. ANDERSON*

U. S. Army Tropical Research Medical Laboratory, San Juan, Puerto Rico

The definitive diagnosis of schistosomiasis depends upon recovery of the schistosome egg from excreta of the infected individual. When this method fails, diagnosis can be made in some instances by demonstrating the egg in tissue biopsied from the liver, rectum or bladder of the patient. Inherent qualities of the infection and limitation of these technics, however, lead to diagnostic failures. Attention has been directed, therefore, to serologic tests for aid in diagnosis of the disease. Complement fixation tests have been described by several investigators.[1-4] Other serologic technics have been described; among these are the cercarienhüllen reaktion,[5] the cercarial agglutination test,[6] the miricidial immobilization test,[7] and the circumoval precipitation test.[8] While all of the serologic procedures aid in the diagnosis of schistosomiasis, methods such as the complement fixation tests require a well equipped laboratory with specially trained personnel. The other methods described rely on a ready source of viable parasitic forms. Need for a simple serologic test was therefore indicated. Slide flocculation technics appear to fulfil that need. However, all attempts to obtain satisfactory reproducible results with the two previously described flocculation tests[9, 10] were unsuccessful at this laboratory. The present report describes a simple slide flocculation test suitable for field and survey purposes.

MATERIALS AND METHODS

Collection and preservation of Schistosoma mansoni *cercariae.* The snail host, *Australorbis glabratus*, either naturally or experimentally infected with *S. mansoni* miricidia, was used as the source of cercariae for these studies. The snails were kept at room temperature and in the dark; food consisted of lettuce and a formula of cerophyl, wheatgerm, glandex, and powdered milk. To obtain cercariae, the infected snails were moved into artificial light for about 6 hours; optimal shedding occurred between the hours of 10 and 12 A.M. Once shed the cercariae were concentrated by sedimentation at 3 to 6° C and were twice washed in distilled water by the same method. The sedimented washed cercariae were transferred to a 10 ml Pyrex ampule and again sedimented in the cold. Following sedimentation, the supernatant fluid in the ampule was reduced to approximately 2 ml by suction from a Richards filter pump. Contents of the ampule were quickly shell-frozen in a mixture of solid carbon dioxide and 95% ethanol (−70° C), and dehydrated under vacuum from the frozen state. After 16 to 18 hours of desiccation the ampule was sealed hermetically under vacuum and stored in a mechanical refrigerator at −20° C.

Cercarial antigen extraction. Prior to extraction of the specific antigen in buffered salt solution, non-specific antigenic lipids were removed from the desiccated cercariae by extraction with cold anhydrous ether. The method used was essentially that of Chaffee *et al.*[4] A 100 mg pool of desiccated cercariae in 10 ml of cold anhydrous ether was ground in a motor driven glass tissue grinder. Both the ether and the grinder had been pre-chilled to −20° C; during grinding the cylinder of the grinder was immersed in a calcium chloride ice-bath (−18° to −15° C). Grinding was continued for 10 minutes after the solid material was in suspension. The ethereal suspension was decanted into a cold glass centrifuge tube and placed in the refrigerator (3° to 6° C). Five ml of cold ether was added to the residual material in the grinder and grinding resumed for 2 minutes. The suspension was combined with the first suspension and the whole centrifuged in a refrigerated angle-head centrifuge (−20° C) at 850 × G for 30 minutes. Immediately thereafter the supernatant ether was decanted and the sediment dried with suction from the filter pump. During the drying process nitrogen gas was blown gently into the tube from a nitrogen

* Major, MSC, USA. Present address: Department of Biology, The Catholic University of America, Washington, D. C.

tank that had been fitted with a reduction valve. When dry, the mass was broken up with a glass stirring rod. The material was returned to the grinder. Quantitative washings of the tube with triethanolamine buffered salt (TBS) solution[11] were transferred to the grinder until 8.0 ml of the TBS had been used. The material was ground for 10 minutes with the cylinder of the grinder immersed in a plain ice-water bath. The suspension was transferred to a cellulose nitrate tube and placed in a refrigerator. An additional 2.0 ml of TBS was added to the residuum in the grinder and grinding was resumed for an additional period of minutes. The 2.0 ml portion was added to the 8.0 ml portion in the cellulose tube and the whole maintained overnight at 3° to 6° C. Following the extraction period, the cellulose tube was centrifuged in a refrigerated centrifuge (3° to 6° C) at 16,000 × G for 30 minutes. The supernate (antigen)* was decanted into a small beaker from which 1.0 ml volumes were pipetted into 10 ml Pyrex ampules. The contents of each ampule were quickly shell frozen and desiccated. The ampules were hermetically sealed under vacuum after 16 to 18 hours of desiccation. As an additional precaution the antigen was stored at −20° C until required for tests.

Serum specimens. Sera from patients proven by stool examination to have *S. mansoni* infections were obtained through the courtesy of physicians at the San Juan City Hospital, Bayamon District Hospital, and the Rodriquez Army Hospital. All of these medical centers are in the metropolitan area of San Juan, Puerto Rico. Sera from non-infected continental donors were obtained through the courtesy of the Department of Serology, Walter Reed Army Institute of Research, Washington, D. C. Continental patients with intestinal nematode infections were used as the source of sera for specificity studies. Specimens of sera from individuals with *Schistosoma haematobium*, *Schistosoma japonicum*, and *Paragonimus westermani* were obtained through the courtesy of Elmer F. Chaffee, Lt. Col., 5th Army Medical Laboratory, St. Louis, Missouri. In addition, the Communicable Disease Center, Chamblee, Georgia, supplied sera from patients with *Trichinella spiralis* infections. Sera in transit were maintained in the frozen state or were preserved with Merthiolate. Otherwise, all specimens were stored at −20° C until required for tests. All sera were inactivated in a water bath at 56° C for 30 minutes just prior to tests. Sera were distributed as unknowns to persons performing the tests.

Selection of a suitable slide flocculation technic. Slide flocculation technics employing cholesterol-lecithin complexes are of two basic types; both types were developed and standardized in the serology of syphilis. One type of test is typified by the Rein-Bossak technic (RB).[12] In this type of test, the antigen emulsion is prepared and then heated at 56° C for 15 minutes. Following the heating, the antigen-cholesterol-lecithin complex is sedimented by centrifugation, the supernate discarded, and the complex resuspended in fresh salt solution. In the other basic type of test[13] (*e.g.*, Venereal Disease Research Laboratory test), the antigen emulsion is prepared, but is neither heated nor washed. Preliminary investigations designed to find a technic suitable for adaptation to schistosomiasis showed that only the former type (RB) of test gave consistent suitable reactions. The latter type (VDRL) yielded variable weak reactions regardless of the numerous ratios of antigen to cholesterol to lecithin used.

An investigation was undertaken to determine if heating, washing or both were essential to the procedure. Four aliquots of an antigen emulsion were treated as follows: (1) not heated and not washed; (2) heated but not washed; (3) not heated but washed; and (4) heated and washed. Each emulsion was then tested against serial two-fold dilutions of the same antiserum. The results obtained led to the selection and development of the procedure presented in the following sections.

Preparation of antigen emulsion. A stock solution of 1.0% cholesterol* and 0.1% lecithin† was prepared in absolute ethanol. The solution was stored at room temperature and in the dark. One ampule of the desiccated antigen was restored to 0.65 ml with distilled water. After the antigen was in solution, 0.6 ml was transferred to a 30 ml round glass-stoppered bottle. The bottom of the bottle was held against the table

* Inoculation of the saline extract of the cercariae into rabbits resulted in the production of antibody detectable by slide flocculation and complement fixation tests.

* Pfanstiehl Laboratories, Inc., Waukegan, Illinois.

† Sylvana Chemical Company, Orange, New Jersey.

top and rotated rapidly, while 0.3 ml of the cholesterol-lecithin solution was added drop by drop. Rotation of the bottle was continued for 10 seconds after the last drop of the solution had been added. The stopper was inserted and the bottle shaken vigorously for 1 minute. Then 1.1 ml of 0.9% NaCl solution was added rapidly and the bottle shaken again for 1 minute. The bottle was allowed to stand for about 30 seconds while the fluid drained from the sides and the emulsion was transferred by a sharp-tipped 1.0 ml pipette to a 15 mm by 100 mm test tube. The test tube was centrifuged at 1000 × G for 15 minutes. The supernate was decanted and the tube held in an inverted position while the sides of the tube were wiped dry with a gauze-wrapped applicator stick. The volume was restored to 2.0 ml with fresh 0.9% saline. When not in use, the antigen emulsion was stored in a refrigerator (3° to 6°C).

Slide flocculation test. For qualitative tests, 0.05 ml of heat inactivated serum was placed in one well of a Boerner glass slide. One drop of antigen emulsion was delivered from a 1.0 ml tuberculin syringe fitted with a 23 gauge needle to each serum to be tested. The needle had been pre-tested to deliver 78 to 82 drops per ml. All tests were machine rotated at 180 rpm for 4 minutes and the results read immediately under low power of a compound microscope. The absence of flocculation was considered as no reaction (−). Where reactions occurred they were recorded as ±, 1+, 2+, 3+, and 4+. Sera that yielded ± or 1+ were considered as weakly reactive (wr); sera giving 2+, 3+, and 4+ flocculation were considered as reactive (R).

Quantitative tests were performed by employing serial two-fold dilutions of serum in 0.9% saline. Each dilution (0.05 ml) was tested in the same manner as that used in qualitative tests. The reciprocal of the last serum dilution showing at least a 2+ reaction was considered as the titer.

TABLE 1

Effect of heating and washing on cercarial slide flocculation antigen

Aliquots of same antigen emulsion	Results obtained with reactive serum dil'd 1:				
	1	2	4	8	16
Not washed					
not heated	2+	±	−	−	−
heated*	2+	−	−	−	−
Washed†					
not heated	4+	3+	2+	1+	±
heated	4+	3+	2+	1+	−

* Heated in a water bath at 56° C for 15 minutes.

† Centrifuged at 1000 × G for 15 minutes, supernate discarded, and the antigen crystals resuspended in fresh salt solution.

TABLE 2

Sensitivity and specificity of the cercarial antigen slide flocculation test

Persons tested		Results obtained in the test*					
Diagnostic status	Number	R		wr		−	
Schistosome infection							
S. mansoni	435	396	(91%)	32	(7%)	7	(2%)
S. japonicum	11	10				1	
S. haematobium	10	6		4			
Other helminthic infections							
T. trichiura	25			1		24	
A. lumbricoides	4					4	
E. vermicularis	2					2	
Hookworm	2					2	
P. westermani	10					10	
T. spiralis	20	11		9			
Healthy	56					56	
Syphilitic	12					12	

* The symbols R (reactive), wr (weakly reactive), − (non-reactive), replace the conventional positive, doubtful, and negative notations.

RESULTS

The results obtained in experiments to show the influence of heating and/or washing upon the sensitivity of the antigen emulsion are presented in Table 1. It is apparent that heating was of no consequence in increasing the sensitivity of the emulsion. On the other hand, washing of the emulsion increased both the degree of the reaction obtained in the undiluted serum and the titer obtained in the serial dilution. Washed antigen emulsions were stable and could be used for at least 6 months if stored at 3° to 6° C when not in use.

Sensitivity of the slide flocculation procedure in *S. mansoni* infections was determined with

sera from 435 patients in whom the disease was confirmed by parasitological examination of the feces. The results of the sensitivity study are shown in Table 2. Sensitivity of the test was considered to be excellent; only 7 (2%) of the 435 patients failed to react in some degree. The incidence of weakly reactive sera was low (7%). Sera from patients with *S. japonicum* and *S haematobium* were tested to determine if infections with other species of schistosomes also produce reactions with the *S. mansoni* antigen. All sera from the 10 patients with *S. haematobium* infections reacted in some degree; sera from 10 of the 11 individuals with *S. japonicum* infections showed a reaction.

The results obtained in specificity studies are also presented in Table 2. All sera from the group of 56 healthy persons showed no reaction; sera from 12 syphilitic patients were negative also. While the number of sera from patients with helminthic infections other than schistosomiasis was relatively small, the group of patients with *Trichuris trichiura*, *Ascaris lumbricoides*, *Enterobius vermicularis*, *Paragonimus westermani*, and hookworm infections showed excellent specificity; only 1 weakly reactive serum was obtained from the 43 patients in the group. On the other hand, the tendency of the cercarial antigen to cross react with *Trichinella spiralis* antibody was apparent. Eleven of the 20 sera tested from these patients showed a reaction and the remaining 9 sera yielded weak reactions.

DISCUSSION

Antigen emulsions that were not washed exhibited a low degree of sensitivity with homologous antibody; washed emulsions, on the other hand, yielded excellent results. While the mechanism of the process is not clear, it appeared that some element or portion of the antigen in the original emulsion had not combined with the cholesterol-lecithin and that the uncombined antigen inhibited the reaction. In experiments, not described here, it was possible to prove the presence of uncombined antigen by using the original supernate as antigen in a complement-fixation procedure. The presence of uncombined antigen could not be attributed to antigen excess in selection of the amount used in preparation of the emulsion as the amount required had been determined by antigen titrations. Use of less antigen in emulsion preparation always resulted in corresponding loss of sensitivity. There are at least two possible explanations for the presence of the uncombined antigen. The first is that the crystals of the emulsion had to be formed in the presence of excess antigen. The second is that more than one antigen was present, one of which would not combine with the cholesterol-lecithin complex but would fix complement in the presence of homologous antibody. Significance of the washing procedure has been emphasized by subsequent findings in other diseases. L. A. Jachowski (personal communication) found the washing procedure essential in slide flocculation tests for filariasis, and the writer (unpublished data) has found similar results with *Nippostrongylus muris* infections in rats.

The occurrence of cross-reactions between the *S. mansoni* flocculation antigen and the sera from patients with *S. japonicum* and *S. haematobium* infections was not surprising. The findings were consistent with those obtained from other soluble antigen tests in schistosomiasis, and emphasize the generic relationship of the organisms.

Cross-reaction of the cercarial schistosome antigen with *T. spiralis* antisera was not unexpected since Liu and Bang[6] reported cross-reactions with living cercaria and *T. spiralis* antisera. Apparently *S. mansoni* antisera do not cross-react with *T. spiralis* antigen adsorbed onto bentonite particles (I. G. Kagan, personal communication). The cross-reaction of the schistosome antigen and the sera from individuals with trichinosis does not seriously handicap the test as a diagnostic tool because geographical distribution of the two diseases is fairly distinct.[14]

SUMMARY

The reaction of cercarial antigen-cholesterol-lecithin complex with schistosome antibody provides a simple sensitive slide flocculation test for schistosome infections.

Capacity of the antigen emulsion to react with homologous antibody was greatly increased by removing the antigen-cholesterol-lecithin complex from the salt solution in which it was prepared and resuspending the complex in fresh salt solution.

The *S. mansoni* cercarial antigen used in the test also reacted with sera from patients with *S. japonicum* and *S. haematobium* infections.

While specificity of the test was excellent in other helminthic diseases, cross-reactions with the *S. mansoni* antigen and *T. spiralis* anti-sera did occur. This finding is consistent with those obtained from other tests using cercarial antigens.

The excellent sensitivity (98%) and simplicity of the procedure make the test ideally suited for field and survey purposes.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. John F. Kent for his encouragement and suggestions in this work; to Capt. Myron G. Radke, USA, for supplying the cercariae used in the study; and to Sgt. Paul B. Broome for technical assistance.

REFERENCES

1. Yoshimoto, M., 1910. Uber die Komplement-bindungsreaktion bie der Schistosomum Krankheit in Japan. *Ztschr. Immunitäts-forsch.*, **5:** 438–445.
2. Fairley, N. H., 1925. Complement-fixation with bilharzia. Part 1. The lipoidal nature of cercarial antigen as used in the complement-fixation test for mammalian bilharziasis. *J. Path. & Bact.*, **28:** 591–607.
3. Miyaji, S., and Imai, B., 1928. Serologische Studien bei Schistosomiasis japonica. *Centralbl. Bakt.*, **106:** 237–246.
4. Chaffee, E. F., Bauman, P. M., and Shapilo, J. J., 1954. Diagnosis of schistosomiasis by complement-fixation. *Am. J. Trop. Med. & Hyg.*, **3:** 905–913.
5. Vogel, H., and Minning, W., 1949. Hüllenbildung bei bilharzia-cercarien im Serum Bilharzia-infizierter Tiere und Menschen. *Centralbl. Bact.*, **153:** 91–105.
6. Liu, C., and Bang, F. B., 1950. Agglutination of cercariae of *Schistosoma mansoni* by immune sera. *Proc. Soc. Exper. Biol. & Med.*, **74:** 68–72.
7. Senterfit, L. B., 1953. Immobilization of *Schistosoma mansoni* miricidia by immune serum. *Proc. Soc. Exper. Biol. & Med.*, **84:** 5–7.
8. Oliver-Gonzalez, J., 1954. Anti-egg precipitins in the serum of humans infected with *Schistosoma mansoni*. *J. Infect. Dis.*, **95:** 86–91.
9. Brandt, J. L., and Finch, E. P., 1946. A simple flocculation slide test for the diagnosis of schistosomiasis. *Am. J. Clin. Path.*, **16:** 141–152.
10. Wright, W. H., Bozicevich, J., Brady, F. J., and Bauman, P. M., 1947. The diagnosis of Schistosomiasis japonica. V. The diagnosis of Schistosomiasis japonica by means of tests. *Am. J. Hyg.*, **45:** 150–163.
11. Kent, J. F., Toussant, A. J., and Hook, W. K., 1957. Adaption of the zymosan assay of properdin to animal sera. *Proc. Soc. Exper. Biol. & Med.*, **96:** 676–678.
12. Rein, C. R., and Bossak, H. N., 1946. Cardiolipin antigens in the sero-diagnosis of syphilis; microflocculation slide test. *Am. J. Syph.*, **30:** 40–46.
13. Harris, A., Rosenberg, A. A., and Riedel, L. M., 1946. A microflocculation test for syphilis using cardiolipin antigen. *J. Ven. Dis. Inform.*, **27:** 169–174.
14. Belding, D. L., 1952. *Textbook of clinical pathology*, 2nd ed., p. 354. Appleton-Century-Crofts, New York.

POLIOVIRUS INFECTION IN GUATEMALA*

MARGARET I. SELLERS,† A. F. RASMUSSEN, JR.‡ AND NEVIN S. SCRIMSHAW§

Physicians in Guatemala have recognized poliomyelitis in sporadic form and in occasional small epidemics for many years, but reported cases are very few. Actual numbers would appear greater than recorded in official vital statistics of the country, because casual observations of rural as well as urban populations reveal frequent paralytic sequelae typical of this disease in all age groups. Such individuals usually state that "paralysis" was first noted during early childhood, commonly following an attack of "bad fever". The single previous report of poliomyelitis in Guatemala, that of Monzón Malice,[1] described an outbreak "que se manifestó en muchos de los departamentos, en proporción tal vez no igualada con anterioridad." Fifty-four cases were reported from the entire country, 28 in Guatemala City. Nearly all were paralytic and 7 were fatal.

Poliomyelitis in Guatemala, as indicated in Table 1, has been less frequent and less severe, although an outbreak among 100 children in an orphanage in early 1959 resulted in 14 (6 paralytic) cases with 3 deaths. Other than the few scattered instances of bulbar poliomyelitis or polioencephalitis, official reports of clinical disease do not specify presence or absence of paralysis. Since no cases of nonparalytic poliomyelitis were reported as such, presumably all were paralytic. The distribution of males and females is essentially equal. Data are not available by age, but children are primarily involved. The occasional paralytic case among children of business and professional people and among foreigners serves to alert private physicians to the dangers of this disease.

As has been established for a number of tropical countries, clinical observations suggest that poliomyelitis infection in Guatemala is far more common than hitherto recognized. To determine this, samples of serum were obtained from 100 adult patients admitted to the Guatemala City General Hospital for conditions unrelated to poliomyelitis. These patients were mainly of low income status and resided in Guatemala City or in nearby towns and villages. Serums were sent to the Department of Infectious Disease, UCLA School of Medicine, where they were tested for presence of neutralizing antibodies to all three types of poliovirus. The results indicate that this group of adults, not known to have had clinical poliomyelitis, had had considerable past experience with all three types of poliovirus.

METHODS

Blood was collected asceptically, the serum separated, samples sent by air to Los Angeles‖ in sterile screw-capped vials and stored at −20° C until examined. Serums were tested for neutralizing antibodies to each of the three types of poliovirus by tissue culture methods employing HeLa cells.[2] Neutralization was determined by direct microscopic examination of cell cultures for cytopathic effect of virus, following the procedure of Syverton, Scherer and Elwood.[3] The test strains of virus were: Mahoney (Type I), MEF-1 (Type II) and Saukett (Type III).

After inactivation of serums in a waterbath at 56° C for 30 minutes, serial dilutions were made in balanced salt solution. Dilutions were 1:4, 1:8, 1:16, 1:64, 1:256, and 1:1024. Aliquots (0.1 ml) of serum dilutions were mixed with 0.1 ml of each of the virus prototypes containing 100 $TCID_{50}$ of virus, and allowed to stand at room temperature for 30 minutes. Next, 0.1 ml of each virus-serum mixture, followed by 0.9 ml maintenance solution containing 20% chicken serum, was transferred to a tube of HeLa cells and incubated at 37° C in a slanting position for 5 to 6 days. Cell cultures were washed with balanced salt solution prior to addition of serum-virus mixture in order to remove human serum incorporated in the growth medium.

Tubes were examined daily and the neutralization titers of the serums were determined by

* INCAP Publication I-144.

† Department of Infectious Diseases, School of Medicine, University of California at Los Angeles.

‡ Division of Virology, School of Medicine, University of California at Los Angeles.

§ Pan American Health Organization, and Institute of Nutrition of Central America and Panama.

‖ Kindly transported by Dr. Edward K. Markell.

TABLE 1

Reported cases of Poliomyelitis, Republic of Guatemala, 1953–1958

Year	Estimated population 1st July*	Number of deaths	Cause specific death rate (× 100,000)	Number of cases†	Incidence per 100,000 population
1953	3058339	23	0.75	not available	not available
1954	3158694	17	0.54	60	1.90
1955	3258010	36	1.10	83	2.55
1956	3346690	37	1.10	70	2.09
1957	3450742	46	1.33	55	1.59
1958	3545901	not available	—	72	2.03

* Dirección General de Estadística (Census Bureau), Guatemala, C. A.

† Dirección General de Sanidad Pública (National Health Department), Guatemala, C. A.

microscopic examination for the dilution at which destruction of HeLa cells by the virus (cytopathic effect) was inhibited by homotypic neutralizing antibody. The controls consisted of retitrations of all the virus types used and the inclusion of known type-specific antiserum neutralization tests against the prototype viruses.

RESULTS

All three types appear to be thoroughly seeded throughout the population. The frequencies of single types of infection with poliovirus and by various combinations of types are given in Table 2. Some 33 persons had neutralizing antibodies to all three types of virus, 34 to two types and 29 to one type of virus only. The serums of only four persons of the 100 failed to reveal neutralizing antibodies to any type of poliovirus in a titer of 1:4 or more. A summary of individual titers of neutralizing antibodies against the three types of poliovirus is recorded in Table 3.

A group of 100 medical students at the University of California at Los Angeles was similarly examined. These subjects were young adults between the ages of 19 and 23. They resided in the Greater Los Angeles urban area and were from families of comparatively high socio-economic status living under high levels of sanitary and public health practices. The results are compared in Table 2 with the Guatemalan serums. The UCLA students gave measurably less evidence of exposure to poliovirus than did the Guatemalans. Nineteen per cent had no antibody, and only 8% had antibodies to all three types of poliovirus.

For further comparison, data from the Guatemalan and the UCLA sera, together with similar data published by Gelfand, Fox and Montoya[4] on sera from an adult urban group of low socio-economic status in Lima, Peru, are given in Table 4. Among the latter individuals, none lacked antibodies of some type and a total

TABLE 2

Frequency of neutralizing antibodies to the viruses of poliomyelitis among young adults of Guatemala and southern California

	Guatemala N = 100	UCLA Students N = 100
No antibodies	4	19
Antibodies to 1 type only	29	42
Type I	22	19
Type II	2	8
Type III	5	15
Antibodies to 2 types only	34	31
I and II	10	12
I and III	11	11
II and III	13	8
Antibodies to all 3 types	33	8

TABLE 3

Titers of poliovirus neutralizing antibody in 100 Guatemalan adults, 1955

Titer*	Type I	Type II	Type III
Less than 4	24	41	38
4	20	13	11
8	13	11	7
16	22	16	16
64	13	11	6
256	2	2	3
1024 or more	6	5	19

* Expressed as the dilution of serum neutralizing 100 $TCID_{50}$ of virus.

TABLE 4

Comparative sero-immunity to poliovirus among groups of individuals in Guatemala, southern California and Lima, Peru

Group age (years)	Number of sera	Percentages of sera with neutralizing antibody					
		Type I	Type II	Type III	Mean†	All types present	No type present
UCLA students (19–23)	100	50	36	42	43	8	19
Guatemalan (20+)	100	76	58	62	65	33	4
Lima, Peru* (20+)	25	96	88	88	91	76	0

* Data presented by Gelfand, Fox and Montoya.[4]

† Mean of the percentages for each type considered separately.[4]

of 76 persons had antibodies against all three types of poliovirus. In general, antibodies to a particular virus type were about two-thirds as frequent in the UCLA series as in the Guatemalan; antibodies among the Peruvian group were uniformly more frequent than in the Guatemalan series.

The data indicate that by the time of young adulthood infection with one or the other of the three types of poliovirus is a common occurrence in Guatemala, despite the relative infrequence of clinically evident paralytic poliomyelitis. Type 1 infection was most common, 76 of 100 Guatemalan adults having had experience with this virus, while 62 had had Type III infection and 58 had had Type II.

DISCUSSION

Since only 100 persons were examined, the results here reported have the recognized limitations of small numbers. They do indicate a greater frequency of poliomyelitis infection in Guatemala than in the United States and certainly show that all three types are well established in the Guatemalan population. At the same time, it is evident that the poliovirus infection rate is only two-thirds as great in the Guatemalan series as in a comparable urban population in Peru; the mean percentage of sero-immunity[4] being 65 for the former and 91 for the latter.

Infection with poliovirus of all three types is also much more common among the population of Guatemala than the known occurrence of clinical disease would indicate. In view of the presumed oral-fecal spread of poliovirus and because of poorer environmental sanitation and habits of personal hygiene among families of the lower social classes, the wide dissemination of poliovirus is not surprising.[5] That overt paralytic poliomyelitis is so rarely seen could indicate (1) that the majority of the individuals exposed come in contact with the virus at an early age when passive immunity from the mother is still present or when other host factors make the occurrence of paralytic diease less likely, or (2) that the prevailing strains of virus are relatively avirulent.[5, 6] Since susceptible non-residents coming to Guatemala, particularly young adults, not infrequently develop paralytic poliomyelitis, it would appear that some wild strains of virus are fully virulent.

As the risk of exposure at an early age lessens in Guatemala under the impact of improved personal hygiene and environmental sanitation, the expectation is that children will not acquire their experience with poliomyelitis virus at as early an age as now, a time when resistance to paralytic disease is high. Paralytic poliomyelitis thus will likely increase along with an increase in age at which antibodies to poliovirus tend to appear. Such changes in the relative frequency of inapparent and overt infection have occurred in many parts of the world as environmental conditions improve. That the above trend is occurring in Guatemala is suggested by the considerable percentage (35%) of the adult population possessing no sero-immunity to the viruses of poliomyelitis. One might anticipate overt disease among these individuals.

The greatest risk at present appears to be among non-residents or transient individuals who previously may not have had contact with these infectious agents. When they contract febrile illness of indefinite nature, poliomyelitis should enter strongly into the differential diagnosis. The evidence of this study suggests that foreigners traveling to Guatemala and similar areas in the tropics and subtropics should be protected in advance by immunization with polyvalent poliomyelitis vaccine.

SUMMARY

The objective of the present study was to determine the frequency of poliovirus infection in Guatemala, primarily the inapparent infections occurring there. Sero-immunity, as indicated by serum neutralization tests in HeLa cell cultures, was demonstrated to all three poliovirus types. Among a group of 100 adults, 76 individuals had antibodies to Type I, 58 to Type II, and 62 to Type III; none of the group was known to have had overt or clinical poliomyelitis. It is concluded that polioviruses prevail in the Guatemalan population to a much greater extent than the occurrence of clinical disease would indicate. It has also been pointed out that 35% of the individuals tested failed to show seroimmunity to poliomyelitis.

Poliomyelitis must be taken into account by the physician in Guatemala in the differential diagnosis of any atypical fever, especially in non-residents. Vaccination with polyvalent poliomyelitis vaccine is highly desirable for foreigners entering tropical and subtropical areas and the probability that vaccination will become increasingly desirable for residents of Guatemala is suggested. The risk of paralytic poliomyelitis is greater than commonly realized.

ACKNOWLEDGMENT

The valuable suggestions of Dr. John Gordon, Professor of Epidemiology, School of Public Health, Harvard University, are gratefully acknowledged.

REFERENCES

1. Monzon Malice, C. M., 1951. Consideraciones sobre el tema de poliomilitis, e informe sobre los casos observados durante el año de 1949. *Rev. Col. med. Guatemala*, **2**: 5–11.
2. Scherer, W. F., Syverton, J. T., and Gey, G. O., 1953. Studies on the propagation *in vitro* of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. *J. Exper. Med.*, **97**: 695–710.
3. Syverton, J. T., Scherer, W. F., and Elwood, P. M., 1954. Studies on the propagation *in vitro* of poliomyelitis viruses. V. The application of strain HeLa human epithelial cells for isolation and typing. *J. Lab. & Clin. Med.*, **43**: 286–302.
4. Gelfand, H. M., Fox, J. P., and Montoya, J. A., 1957. Survey for seroimmunity to poliomyelitis in Peru and Colombia. *J. Trop. Pediat.*, **3**: 51–61.
5. Paul, J. R., 1955. Epidemiology of poliomyelitis. *World Health Organ., Monog. Ser.*, No. 26: 9–27.
6. Gear, J. H. S., 1955. Poliomyelitis in the underdeveloped areas of the world. *World Health Organ., Monog. Ser.*, No. 26: 31–58.

THE EFFECT OF FOLIC ACID ON THE STEATORRHEA OF TROPICAL SPRUE AND OTHER TESTS FOR INTESTINAL ABSORPTION*

R. RODRIGUEZ-MOLINA,† MARTA CANCIO‡ AND CONRADO F. ASENJO§

San Juan, Puerto Rico

Tropical sprue is not infrequently encountered in Puerto Rican male war veterans, particularly those over 40 years of age. The various clinical aspects of this condition have been intensively studied, and it has been found that an abnormal loss of fat in the stools is one of its most characteristic and constant features. Rodríguez-Molina *et al.*[1] and C. F. Asenjo *et al.*[2] have carried out fat-balance studies in patients affected with this condition. All their cases showed a steatorrhea of exogenous origin resulting from a fat absorption defect rather than from an increased excretion of endogenous fecal fat. When these patients received a low-fat diet (under 10 g daily), their endogenous fecal fat excretion was of about the same order of magnitude as that of normal subjects.

Suárez *et al.* in 1947,[3] established that folic acid is an efficient agent in the treatment of tropical sprue as it occurs in Puerto Rico. Weakness, glossitis, lassitude, diarrhea and anemia improve under treatment. It also produces improvement of the patient even in the absence of anemia or clinical evidence of secondary nutritional deficiencies.[4]

So far as we know, controlled fat-balance studies in tropical sprue have not been carried out to determine the effect of folic acid on the malabsorption defect, as indicated by the presence of steatorrhea and by the results of other tests for intestinal absorption, such as the oral glucose tolerance, vitamin A tolerance and D-xylose excretion test. The present study was undertaken to determine the action of the drug in patients showing steatorrhea and in whom other tests for intestinal absorption were observed to be abnormal prior to treatment.

* This investigation was carried out as a cooperative project under a grant from the U. S. Public Health Service, National Institutes of Health, Research Project A-889.

† General Medical Research Laboratory, Veterans Administration San Patricio Hospital, and School of Medicine and School of Tropical Medicine, University of Puerto Rico.

‡ General Medical Research Laboratory, Veterans Administration San Patricio Hospital.

§ Department of Biochemistry and Nutrition, School of Medicine and School of Tropical Medicine, University of Puerto Rico.

MATERIALS AND METHODS

To determine the presence and degree of steatorrhea in a sprue patient it is necessary to administer a standard diet containing a constant amount of digestible fat in addition to other foodstuffs, and to determine the total daily output of fat in the feces. It was decided to adopt the Mayo Clinic steatorrhea test diet[5] with some modifications. The reader is referred to a previous communication for further details.[2] The latter consisted of fruit juices, dry breakfast cereals, Cream of Wheat, white bread, potatoes, three different salads of lettuce with fruits and dressings, cooked rice, jelly, baked apple, water-packed fruits, string beans, carrots, one or two eggs, lean beef or lamb, butter and 1 liter of milk per day. Three menus were made up from these food items which, if fully consumed, supplied between 90 to 100 grams of fat and about 10 grams of gluten per day. Some patients received the Mayo Clinic diet containing about 40 grams of gluten, an amount which, in our experience, has had no effect on the steatorrhea.

A dietitian specially assigned to this study prepared and served the food. This was weighed out before serving. A whole day's serving was homogenized, and aliquots of this homogenate were analyzed for total fat and solids. In order to make the necessary corrections, samples of the residue left by each individual were also analyzed. The patients were kept under strict surveillance. Stools were collected and weighed daily, stored in the ice box and within 48 hours homogenized by the addition of a known amount of water, if necessary, and analyzed for total fats. Dietary and stool fat determinations were made by the method used by Van de Kamer *et al.*,[6] described in a previous communication by the present authors.[2] Since by this method the percentages of total fatty acids in the diet and in the feces are known, as well as the weight of diet ingested and feces excreted per day, the intake and output per

TABLE 1

Results of fat balance studies on twelve patients prior to and during treatment with folic acid*

Patient*		Fat content in grams per 24 hrs									
		Before treatment					During treatment				
No.	Color & Age	In diet		In stools		Days of tests	In diet		In stools		Days of tests
		Mean	S.D.†	Mean	S.D.		Mean	S.D.	Mean	S.D.	
1	W 63	66	23	6	2	15	78	22	8	4	16
2	W 70	66	23	28	16	15	81	24	17	16	18
3	W 35	66	12	16	7	15	80	44	14	15	15
4	W 70	101	40	10	4	15	84	23	13	6	12
5	C 63	117	16	49	21	15	83	13	26	18	19
6	W 62	68	15	20	8	21	68	11	17	4	16
7	C 63	78	21	35	15	21	76	17	40	20	42
8	W 70	72	9	8	4	21	85	17	8	5	36
9	C 62	60	20	8	3	6	94	39	7	5	22
10	W 63	50	18	17	6	3	82	28	5	5	44
11	W 63	83	34	12	7	20	112	38	16	9	9
12	W 52	105	52	11	7	36	106	33	17	6	23
Mean		78	24	18	8	17	86	26	16	9	23

* Patients 1, 3 and 6 were acutely ill; all others were in a state of chronic relapse. All except No. 11 showed improvement following folic acid administration.

† Standard deviation.

day of total fat, in terms of fatty acids, were calculated. An amount of fat over 6 grams in the 24 hour stool was considered abnormal.

Other tests for intestinal absorption such as oral glucose tolerance, vitamin A absorption and D-xylose excretion were performed, according to standard procedures.[7-11]

Red cell and hemoglobin determinations were performed according to methods described by Wintrobe[12] and Hainline.[13]

Cases. Ten individuals were included in the study, eight white and two colored. With the exception of two patients, aged 35 and 52, all were over 60 years of age and were born in Puerto Rico of Spanish or Negro extraction. Previous fat-balance studies under folic acid treatment were repeated in two cases (Numbers 4 and 8, 5 and 7 in Table 1), after an interval of 1 year when the illness was in a stage of chronic relapse. Three other cases (Numbers 1, 3, and 6) were hospitalized in the acute phase of the first attack; symptoms lasted from 1 to 3 months and no treatment was given. All other patients had suffered from the disease for periods of 6 months to 40 years prior to the time of the study, and had had several remissions induced by special diet, liver extract, folic acid or vitamin B_{12}.

Diagnosis. Diagnostic criteria included the following:[14] insidious onset, chronic symptoms and infrequent spontaneous remission; gastro-intestinal disturbances (dyspepsia,* diarrhea, and steatorrhea); stomatoglossitis, with or without atrophy of the lingual papillae; early weight loss accompanied by increasing weakness and prostration; free hydrochloric acid in the stomach (in over 90% of the cases); macrocytic, hyperchromic anemia associated with a megaloblastic marrow and absence of neurologic manifestations in about 90% of the cases. Laboratory findings are a flat glucose tolerance curve; low vitamin A tolerance test (values below 75 mg in 5 hours and below 125 mg in 7 hours); urinary D-xylose excretion below 3.0 g within 5 hours; blood serum vitamin B_{12} levels lower than 200 micromicrograms per ml following ingestion of food; and a daily fat level in the 24 hour stools of over 6 grams.

* This term comprises, in order of frequency, abdominal distention, epigastric distress (not necessarily related to the taking of food), heartburn, and generalized abdominal discomfort and pain.

TABLE 2

Results of oral glucose tolerance test in two patients showing improvement after treatment with folic acid

Time of test	Blood glucose, mg/100 ml			
	Patient 3		Patient 8	
	Before treatment	After treatment	Before treatment	After treatment
Fasting	85	75	94	81
½ hour	89	122	105	150
1 hour	82	83	127	96
2 hours	67	86	111	39
3 hours	76	57	89	33

TABLE 3

Results of oral vitamin A tolerance test in five patients

Patient no.	Plasma vitamin A, micrograms/100 ml					
	Before treatment			After treatment		
	Fasting	5 hrs	7 hrs	Fasting	5 hrs	7 hrs
3	48	72	108	72	120	125
7	45	60	56	83	63	100
8	54	60	114	110	110	131
9	62	77	80	209	204	205
10	68	88	129	154	171	161

TABLE 4

Results of D-xylose absorption test in six patients, as shown by amounts excreted in the urine during 5 hours following oral administration

Patient no.	Grams of D-xylose excreted	
	Before treatment	After treatment
3	1.6	2.7
6	1.2	2.5
7	0.6	1.4
8	0.7	1.2
9	1.0	1.2
10	2.7	0.4

Other tests for intestinal absorption. The oral glucose-tolerance test was performed in eight cases, the D-xylose absorption test in six, and the vitamin A tolerance test in five. All tests were done both prior to and following administration of folic acid.

Diet. The diet, which was quite palatable, contained 50 to 100 grams of easily digestible animal and vegetable fats daily. In addition, it contained 80 to 85 grams of animal protein and about 200 grams of carbohydrate, a total of 2,000 to 2,200 calories. It was given during a period of observation averaging 17 days, and continued over periods of therapy lasting an average of 23 days.

Study method. Daily fat-balance studies before administration of folic acid ranged from 3 to 36 days, averaging 17 days for the group of patients under study. The period of treatment lasted from 9 to 44 days with an average of 23 days.

Treatment consisted of 15 mg folic acid given daily, in three doses of 5 mg each, taken by mouth immediately after meals. Case 12 was given a similar amount of drug in a single intramuscular injection daily. In each case administration of drug was begun on the day following the last day of the observation period.

RESULTS

Fat-balance studies: Results of twelve fat-balance studies in the ten subjects are shown in Table 1. Severe steatorrhea (over 12 g) was observed in seven cases (2, 3, 5, 6, 7, 10 and 11) during the period of observation prior to treatment. Mild steatorrhea (less than 12 g) occurred in five patients (1, 4, 8, 9 and 12) during this period. During the period of folic acid administration severe steatorrhea was noted in eight cases (2, 3, 4, 5, 6, 7, 11 and 12), while no steatorrhea or only mild steatorrhea was observed in four cases (1, 8, 9 and 10) during this period.

Patients 2, 3, 5, 6, 7, 10 and 11 also showed severe steatorrhea prior to therapy, while in patients 4 and 12 mild steatorrhea had been observed.

Oral glucose tolerance test: No change was observed in five cases. That is, a flat curve was unaffected by treatment. Two patients gave normal levels and the eighth became worse after treatment. In the patients showing improvement, results were as given in Table 2.

*Oral vitamin A tolerance test:** Improvement was observed in four of the five cases in which the test was performed. Values after treatment

* Normal values for vitamin A tolerance are as follows: A serum level of at least 75 mg % of vitamin A at 5 hours, and 125 at 7 hours after an oral dose of 300,000 units of vitamin A.[5]

TABLE 5

Red blood cells and hemoglobin before and after folic acid therapy

Patient no.	Before treatment		After treatment		Days treatment	Change	
	Millions RBC/cmm	Hb grams %	Millions RBC/cmm	Hb grams %		RBC	Hb
1	2.90	9.4	3.4	13.0	16	0.50	3.6
2	2.45	10.2	3.8	12.2	18	1.35	2.0
3	2.64	9.3	3.5	11.4	15	0.86	2.1
4	2.4	7.0	3.00	8.5	12	0.60	1.5
5	3.6	11.0	4.00	11.6	19	0.40	0.6
6	2.3	7.0	3.91	10.4	16	1.60	3.4
7	2.66	10.7	4.11	12.9	42	1.45	2.2
8	2.30	6.9	3.7	10.7	36	1.40	3.8
9	4.39	12.6	4.00	12.6	22	.39	0.0
10	1.70	7.6	3.9	11.0	44	2.20	3.4
11	3.70	11.3	3.9	11.4	9	0.20	0.1
12	2.90	8.8	3.6	11.7	23	0.70	2.9

reached normal levels in these patients. The other case presented normal figures even prior to treatment (Case 10). Results are shown in Table 3.

D-xylose absorption test:† Although some improvement was observed in five patients, results after treatment were below normal levels. Case 10 showed less excretion following therapy (Table 4).

Red cells and hemoglobin: Improvement was observed following treatment (case 11 excepted) although levels reached were subnormal. Patients 7 and 10 who were treated longer than the others (42 and 44 days) still did not attain normal values, but on the other hand after only 16 days of treatment case 6 had reached similar levels (Table 5).

Course in the hospital. Clinical improvement under folic acid was noted in all patients except one, case 11, who was discharged from the hospital at his own request after only 9 days of treatment. All others showed improvement in general condition, glossitis, and diarrhea. Gain in weight was observed in some but, generally speaking, the patients did not respond markedly to treatment.

DISCUSSION

When the mean values for daily fat output in the stools before and during treatment were considered individually for each of the twelve patients, the following results were observed: No change in the amount of steatorrhea during the period of treatment was noted in two patients (Cases 8 and 9). In five cases, less steatorrhea was observed during therapy than before treatment (Cases 2, 3, 5, 6, and 10). In five other patients more severe steatorrhea appeared to have occurred during the period of therapy than during the period of observation prior to treatment (Cases 1, 4, 7, 11 and 12). (Figure 1.)

However, when the data were subjected to statistical analysis and the significance of a difference between means (before and during treatment) was measured for each case, it was found that in eight patients (Cases 2, 3, 4, 6, 7, 8, 9 and 11) it was probably not significant. This indicates that folic acid did not produce a significant change in the steatorrhea.

In cases 5 and 10 the difference between means was probably significant, indicating less steatorrhea during treatment than during the period of observation (49 g reduced to 26; 17 to 5.0). Cases 1 and 12 also showed a probable significant difference between means but this pointed to an increase in steatorrhea during treatment (from 6 to 8 g; 11 to 17).

Finally, when results were analyzed for the twelve patients as a unit and the significance of the difference between the means (18.4 and 15.8 g; difference: 2.6 g) of daily fat output in the stools, prior to and during therapy, was measured it was ascertained that it was probably not significant.[15] This indicates that under the conditions of the experiment folic acid ap-

† The test measures the xylose excreted in total urine within 5 hours after ingestion of the sugar. Excretion of 3.0 g or more after an oral dose of 25 g of D-xylose is considered normal.[9-11]

STEATORRHEA IN TROPICAL SPRUE — MEANS OF DAILY (24 HOUR STOOLS) OUTPUT BEFORE AND DURING TREATMENT FOR EACH OF 12 CASES OF SPRUE

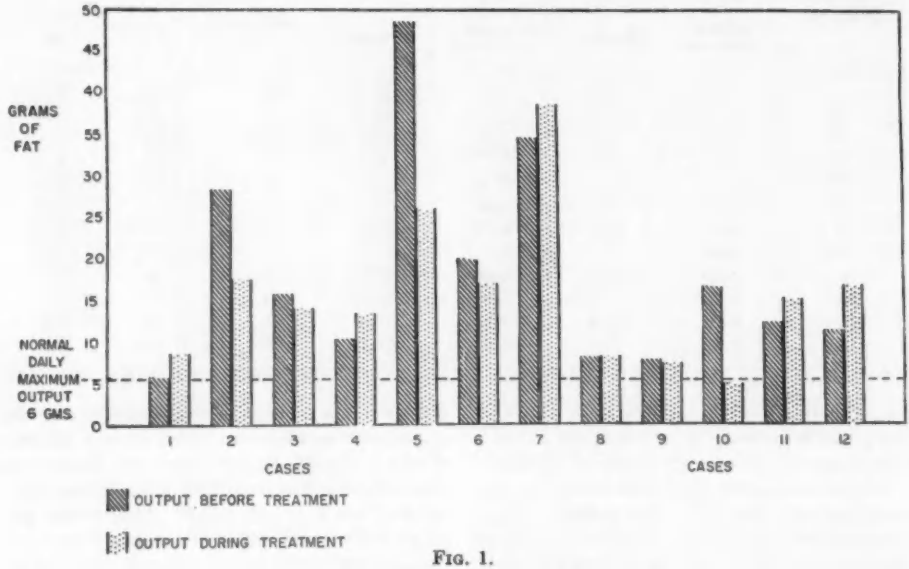


FIG. 1.

FAT BALANCE STUDY PRIOR TO AND DURING TREATMENT OF SPRUE WITH FOLIC ACID

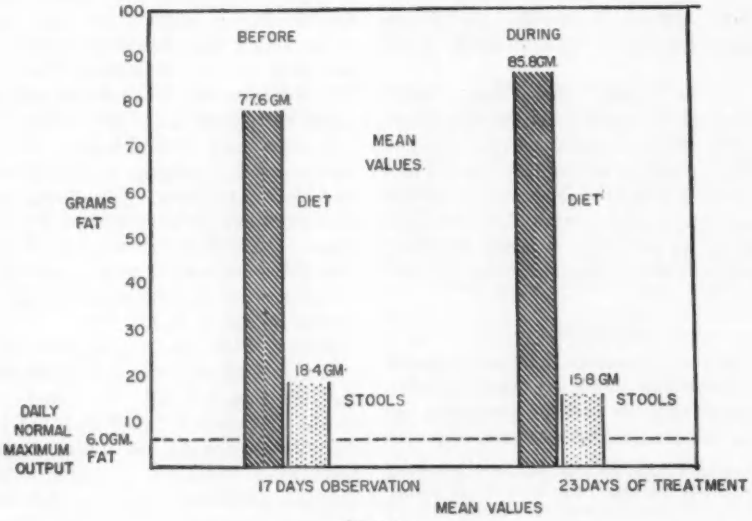


FIG. 2.

peared to have had no effect on the steatorrhea. (Figure 2).

The hematologic response to folic acid treatment of a sprue patient is considered a reliable index of the beneficial action of the drug. The mean increase of red cells for the group of cases during the treatment was one million with 2 grams of hemoglobin. In our experience such hematologic response is far from being dramatic or even adequate. Striking results have been observed in younger individuals with similar doses of folic acid. The advanced age of the majority of patients and the comparatively short time (average 23 days) of therapy may have been the factors responsible for the poor results in these patients. The minimal effect of folic acid revealed by the fat-balance studies and other tests for intestinal absorption have paralleled the weak effect on red cell and hemoglobin production.

Case 10 showed normal values for vitamin A absorption prior to treatment. The glucose tolerance test was also normal before treatment (78; 154; 174; 153; 114 mg %), but the excretion of D-xylose was only 2.78 grams during this period, and worse (0.4 g) after folic acid administration. Fat-balance studies demonstrated a significant reduction in steatorrhea during treatment (16.6 to 4.97 g).

It appears that length of treatment per se is not the only factor responsible for inadequate hematologic response. This was brought out in patients 7 and 10 (ages 63 and 63), who were treated longer than the others (42 and 44 days) and still did not attain normal red cell and hemoglobin values. On the other hand after only 16 days of treatment case 6 (age 62) had reached similar levels.

SUMMARY

1. Twelve fat-balance studies were performed in ten documented sprue patients before and during the administration of 15 mg folic acid given orally and daily over a period ranging from 9 to 44 days, with an average of 23 days for the twelve cases. All patients showed steatorrhea during a period of observation before treatment.

2. Other tests for intestinal absorption such as glucose tolerance, D-xylose excretion, and vitamin A absorption were carried out prior to and after treatment in some cases.

3. Steatorrhea was significantly reduced during treatment in two patients, and it was increased in two other cases. In the remaining eight, folic acid did not produce any significant changes in the steatorrhea.

4. Vitamin A absorption improved in four cases and glucose tolerance in two cases. Results of other tests for intestinal absorption (glucose tolerance, D-xylose excretion and vitamin A absorption), though slightly improved, remained below normal values in other patients.

5. Under folic acid therapy all but one patient showed improvement in general condition, in glossitis, in weight, and in red cells and hemoglobin. The hematologic response to the drug, however, was inadequate in all cases.

6. Probable factors responsible for the poor effect of folic acid on the blood, on steatorrhea and on the malabsorption of glucose, D-xylose and vitamin A are discussed.

REFERENCES

1. Rodriguez-Molina, R., Asenjo, C. F., and Cancio, M., 1957. Steatorrhea in tropical sprue patients in Puerto Rico. *Am. J. Trop. Med. & Hyg.*, **6:** 780–785.
2. Asenjo, C. F., Rodriguez-Molina, R., Cancio, M., and Bernabe, R. A., 1958. Influence of very low-fat diets with and without gluten on the endogenous-fecal-fat excretions of patients with tropical sprue. *Am. J. Trop. Med. & Hyg.*, **7:** 347–352.
3. Suárez, R. M., Spies, T. D., and Suárez, R. M., Jr., 1947. Use of folic acid in sprue. *Ann. Int. Med.*, **26:** 643–677.
4. Gardner, F., 1958. Sprue. In *Principles of Internal Medicine*, Harrison *et al.*, 3rd ed., pp. 529–533. McGraw-Hill Book Co., New York.
5. Mayo Clinic, 1954. *Diet Manual*, 2nd ed. W. B. Saunders Co.
6. Van de Kamer, J. M., Ten Bokkel Huinink, R., and Weijers, H. A., 1947. Rapid method for the determination of fat in feces. *J. Biol. Chem.*, **177:** 347–355.
7. Folin, O., and Wu, H., 1953. Glucose. In *Standard Methods of Clinical Chemistry*, vol. 1, pp. 60–64. Academic Press, Inc., New York.
8. May, C. D., Blackfan, K. D., McCreary, J. F., and Allen, F. H., Jr., 1940. Clinical studies of vitamin A in infants and children. *Am. J. Dis. Child.*, **59:** 1167–1184.
9. Butterworth, C. E., Jr., and Perez-Santiago, E., 1959. Studies on the oral and parenteral administration of D(+)xylose. *New England J. Med.*, **261:** 157–164.
10. Butterworth, C. E., Jr., Perez-Santiago, E., and Santini, R., Jr., 1959. Xylose. In *Tropical Sprue. Studies of the U. S. Army's Sprue Team in Puerto Rico*, ed. by W. H. Crosby, p. 73. Medical Science Publication No. 5, Walter Reed Army Institute of Research, Washington, D. C.
11. Perez-Santiago, E., and Butterworth, C.

E., Jr., 1959. Definition and diagnosis of sprue. In *Tropical Sprue. Studies of the U. S. Army's Sprue Team in Puerto Rico*, ed. by W. H. Crosby, p. 101. Medical Science Publication No. 5, Walter Reed Army Institute of Research, Washington, D. C.

12. Wintrobe-Maxwell, M., 1956. *Clinical Hematology*, 4th ed. Lea and Febiger, Philadelphia.
13. Hainline, A., Jr., 1958. Cyanmethemoglobin method. In *Standard Methods of Clinical Chemistry*, vol. 2, p. 52. Academic Press, Inc., New York.
14. Rodriguez-Molina, R., 1954. Fundamental concepts in the diagnosis of sprue. *Ann. Int. Med.*, **40**: 33–41.
15. Davenport, C. B., and Ekos Merle, P., 1956. *Statistical Methods in Biology, Medicine and Psychology*. John Wiley & Sons, Inc., New York.

INFECTIVITY TO MOSQUITOES OF *PLASMODIUM VIVAX* AND *PLASMODIUM FALCIPARUM* UNDER VARIOUS CONDITIONS*

GEOFFREY M. JEFFERY†

Some of the most interesting studies in human malaria in which I have participated have concerned factors of importance in the epidemiology of the disease, especially the conditions under which the infection is transmissible to mosquitoes, and the possible alteration of this transmissibility through the judicious selection and use of antimalarial drugs. One who is engaged in this field of research soon discovers that these conditions can be quite complex and puzzling. For example, the correlation between gametocyte densities in the human host and the infectivity to mosquitoes may be extremely variable, depending on the stage of the infection, the species —or even the strain—of parasite, and many other factors. It is felt that an understanding of these conditions is of considerable importance as an aid to the efforts now underway toward eradication of the disease.

The present report will deal first with infectivity of the malaria infection to mosquitoes at various stages of the infection in man, and second with the infectivity after treatment of the disease with various antimalarial drugs. Much of the work forming the basis for this report has already been published but some data are being reported for the first time. Most of the studies included here were undertaken with the epidemiology of the infection in mind. That is, it was desired to know at what points in the course of infection in the human host were our defenses against mosquito transmission weak or faulty. For instance, could transmission of malaria in endemic areas take place if case-finding and treatment were limited only to clinical cases; and can we depend on the antimalarial drugs in use at present to eliminate quickly the carrier as a source of infection? These and other questions can be at least partially answered with the data at hand.

The discussions will be limited to the Chesson strain of *Plasmodium vivax* and three strains of *P. falciparum:* one from Panama and two from South Carolina.

STAGE OF INFECTION IN MAN

Considering first the relationship between the stage of the malaria infection and infectivity to mosquitoes, the infection in man can be conveniently divided into several periods:

1. Early (preclinical) parasitemias.
2. Clinical period.
3. Terminal (asymptomatic) parasitemias.
4. Relapses (asymptomatic and symptomatic).

1. *Early parasitemias.* This period falls between the first appearance of parasites in the peripheral blood and the time when frank malaria symptoms appear. Its significance in the epidemiology of malaria is obvious: Where treatment is not initiated until symptoms appear, a preceding infective period would be of some importance.

P. vivax: A considerable amount of work has been done on the early infectivity of this species and the results have been published.[1] In a group of 24 patients mosquito feedings were done from the first day of parasites as long as practicable or until suppressive treatment intervened. Mosquitoes became infected an average of 4 days after appearance of first parasites and on parasite densities averaging about 1500 per cmm of blood. In all cases but one, mosquitoes became infected before gametocytes were demonstrable by the normal methods used (30 to 35 per cmm of blood). Even when larger amounts of blood were examined, it was quite evident that in many cases mosquitoes became infected when less than 10 gametocytes per cmm of blood were present. Of the 24 cases, none infected mosquitoes prior to first recorded temperatures in excess of 100° F, but all did so prior to temperatures exceeding 104° F. Thus, although lower grade fevers had usually appeared prior to or at about the same time as infectivity, the first frank malarial paroxysm usually had not occurred. Certainly the

* Address given at the Dinner Session of the American Society of Tropical Medicine and Hygiene on October 30, 1959, by the recipient of the Bailey K. Ashford Award, provided through the generosity of the Eli Lilly Company.

† Department of Health, Education, and Welfare, Public Health Service, National Institute of Allergy and Infectious Diseases, Laboratory of Parasite Chemotherapy, P. O. Box 717, Columbia, South Carolina.

fever pattern characteristic of the malaria attack had not yet emerged.

It is concluded that the early parasitemias of *P. vivax* are of epidemiological importance. Even in areas where good therapeutic measures are available, the primary attack could provide a reservoir for infection before it could be terminated or suppressed.

P. falciparum: The work that has been done on the early parasitemias of this species leave little doubt that the "pre-clinical" period is of little importance in the mosquito transmission of the infection. In over 150 cases of three strains of falciparum, gametocytes appeared a mean of 11.5 days after first asexual parasites, while first significant fevers appeared after a mean of about 2 days of parasites.[2] Mosquito feedings done on the early parasitemias in 10 cases resulted in no mosquito infections prior to the detection of gametocytes by normal methods (10 per cmm or more), and the mean day for first mosquito infections was the 3rd day of patent gametocytes. Thus, it is quite evident that the early parasitemias of *P. falciparum* are not of epidemiological importance in the same sense as those of *P. vivax*. There would be no possibility of mosquito infections previous to the clinical attack and the clinical attack is generally of such severity as to warrant curative treatment where such is available. Probable efficacy of this treatment in interrupting transmission will be considered a little later.

2. *Clinical period.* Most of the mosquito feedings which we have done over the years in our experimental program have been done during this period, and although the results are quite voluminous they are also somewhat puzzling, especially in regard to *P. vivax*.

P. vivax: After the appearance of symptoms and through the course of the clinical attack one generally finds fairly consistent infectivity to mosquitoes. However, there seems to be much variation between individual patients, some being what we call good infectors and some very poor. None of our work has produced a reasonable explanation for this phenomenon. Also quite evident in some cases are waves of infectivity, where periods of a few days of good infectivity to mosquitoes would be followed by periods of poor or no infectivity. Here again no reasonable explanation has emerged; it has not been possible to correlate these periods with total parasite densities, gametocyte densities, febrile activity, or other characteristics of the infection, and no morphological differences in the parasites are apparent.

A total of over 500 mosquito feedings done on 56 sporozoite-induced *P. vivax* infections during the first 35 days of parasites reveals some interesting results. A very definite early wave of high infectivity occurs on or about the 7th day of parasites. Following this the infectivity declines, remaining fairly low until about the 13th day of parasites, when it usually becomes extremely high for about 10 days. Following this period the rise and fall of infectivity is extremely variable in individual cases and little pattern could be noted from the smaller number of mosquito feeding experiments done.

It is clear that this clinical period is one of extreme danger so far as the transmission of *P. vivax* is concerned, and it is this period which would be concerned with the role of drugs in the interruption of transmission.

P. falciparum: The clinical period in this species includes all of the primary parasitemia following the advent of the clinical attack, including the period of asymptomatic parasitemia which may continue for a time after the cessation of symptoms. Usually gametocytes appear in detectable numbers (10 per cmm or more) on the 10th to 13th day of parasite patency, and are infective to mosquitoes on or about the 3rd day of gametocyte patency.[2, 3] The increase in gametocyte densities to high levels usually predicts a decrease in the asexual parasite density, and the symptomatic attack may terminate. Gametocytemias may persist for periods of several months without interruption. Infectivity to mosquitoes, once it is initiated, usually will continue at high levels as long as the gametocytes continue uninterrupted either spontaneously or by drug. While the infectivity of this species is considerably more predictable than *P. vivax*, even here there are individual variations between patients which cannot be correlated with any other known factor. Some patients are exceptionally good infectors, while occasionally a patient will infect very few mosquitoes even with presumably adequate gametocyte densities.

The danger of transmission of *P. falciparum* during this initial period of continuous parasitemias is indeed great, but, because it begins with a very apparent period of symptomatic

malaria, interruption of transmission by drug may in practice be accomplished more frequently than during a period of apparently less efficient transmission.

3. *Terminal (Asymptomatic) Parasitemias.* This period applies only to *P. falciparum* and appears to be a characteristic of this species. Practically every *P. falciparum* infection, if treated inadequately or not at all, will continue to show parasitemias intermittently for long periods after the termination of the primary continuous parasitemia. This terminal period averages about 168 and 100 days for the Panama and Santee-Cooper strains, respectively, with a mean total persistence of the infection of 279 and 222 days. Longest persistence for the two strains was 503 and 480 days.[4] Infection of mosquitoes was frequently successful during this terminal period; in both strains about one-third of the lots fed on these low-grade parasitemias became infected. Latest infectivity was seen at day 321 in the Santee-Cooper and day 410 in the Panama strain. Others have shown that these asymptomatic parasitemias are infective under field conditions. Young *et al.*,[5] were able to infect 41 per cent of the lots of mosquitoes fed at random on asymptomatic carriers of *P. falciparum*; indeed 4 per cent of the lots fed on persons not exhibiting symptoms or demonstrable parasites (<10 per cmm of blood), but with history of the disease, became infected. The epidemiological significance of these late infective parasitemias is quite obvious: Their long continuance may be responsible for renewal of transmission during new malaria seasons, as well as providing a continuing source of infection in an endemic area. Adding to their importance is the consideration that these parasitemias are not usually accompanied by symptoms, so that they would only be detected in mass surveys; further, because of their intermittent character many of them would not be detected even by this method.

4. *Relapses:* This period is limited to *P. vivax*, of which species the relapse is characteristic. There is adequate evidence that relapsing vivax parasitemias are potentially dangerous as infectors of mosquitoes,[6, 7] and we have been able to confirm these results. In our experience all symptomatic relapses have been infective to mosquitoes; mosquitoes invariably became infected during the early days of the relapse, usually at least 3 to 4 days before gametocytes were present in detectable numbers, and usually before the onset of fever. Infectivity of relapsing parasitemias appears to be much better than in comparable primary parasitemias. High incidence and intensity of infection often persist through the course of the relapse, even after symptoms cease and the parasite densities become very low. Our limited studies have not shown that parasite relapses unaccompanied by symptoms, which sometimes occur in the Chesson strain, are infective to mosquitoes. Others[7] have demonstrated infectivity to mosquitoes of these asymptomatic relapses. The special epidemiological significance of the *P. vivax* relapse is associated with the early infectivity of these relapsing parasites and the continued infectivity of the low grade parasitemias with minimal symptoms or after cessation of symptoms.

In summary, the infectivity of untreated or inadequately treated *P. falciparum* to mosquitoes is generally intense during the primary parasitemias, which generally continue for 3 to 4 months. An even longer period of low grade intermittent parasitemias, though admittedly less infective to mosquitoes, is probably of even greater importance in the maintenance of the disease in endemic areas and in the carrying over of the transmission from one malaria season to another. In *P. vivax*, an early preclinical period of infectivity is of importance in perpetuation of transmission despite treatment of clinical cases. The primary parasitemias are, of course, of great importance when they are not treated adequately. Continuing relapses, infective to mosquitoes, provide the mechanism for season-to-season transmission and for the presence of infective, asymptomatic parasitemias in an endemic situation.

POST-TREATMENT INFECTIVITY

The normal pattern of infectivity to mosquitoes can be altered through the use of antimalarial drugs, cure of the infection, or by sporontocidal action in the absence of cure. Much progress has been made in the use of drugs in the interruption of mosquito transmission, and in this field lies much of the ammunition for malaria eradication. For this reason some of the work on the effect of drugs on infectivity to mosquitoes might well be reviewed here.

1. *Chloroquine*: The curative action of chloroquine against *P. falciparum* is well known, as is its frequent failure to prevent relapses of *P. vivax*.

Ironically enough, even though it is curative for *P. falciparum* it has no immediate effect on the presence or infectivity of gametocytes in this species; on the other hand while it does not usually prevent relapses of *P. vivax*, it does almost immediately remove infective gametocytes from the blood.

In 29 cases of falciparum treated with a routine 1.5 grams of chloroquine gametocytemias were seen to persist as late as 26 days after treatment, and mosquitoes could be infected as late as 20 days post-treatment.[8] It is probable that infectivity would persist as long as gametocytes remain in sufficient numbers. When one considers that the patient would be ambulatory before these infective gametocytes disappeared from the blood, the epidemiological significance is obvious.

The question as to how early chloroquine must be given to prevent the development of gametocytes of *P. falciparum* is of some importance. In 17 cases, giving the drug on or before the 6th day of parasites prevented the development of gametocytes; in one of two cases at 7 days and two of three at 8 days gametocytemias were also prevented. In all cases where the drug was given after 9 or more days of parasite patency gametocytes developed normally, even though asexual parasites were rapidly eliminated.

Treatment of *P. vivax* with chloroquine usually removes the infective gametocytes within 2 days after treatment; in most cases mosquitoes can be infected 24 hours after treatment, and in a very few 48 hours after treatment.[9] While chloroquine appears to have considerable efficiency as a sporontocidal agent in vivax malaria, it should be recalled that relapses frequently occur, with the accompanying problem of parasitemias of high infectivity to mosquitoes.

2. *Chlorguanide:* The use of chlorguanide as a sporontocidal agent in *P. falciparum* has been well elucidated by Fairley *et al.*, [10] Shute and Maryon,[11] Covell *et al.*, [12] Ramakrishnan *et al.*,[13] and others. Dosages as low as 100 to 300 mg usually rendered the patient non-infective to mosquitoes for periods of 2 to 7 days, but infectivity returned very rapidly to high levels following this period. It is of interest that chlorguanide does not usually prevent the development of gametocytes when given fairly early in the attack. In 7 cases treated with 300 mg on the 6th to 9th day of parasites gametocytes appeared on schedule and in normal numbers. Lower dosages given even earlier had no discernible effect. The question of strains of malaria resistant to chlorguanide is of importance. It has been demonstrated[14] that gametocytes of resistant strains of *P. falciparum* continue to infect mosquitoes during and after administration of the drug, confirming the extension of resistance to the gametocytes.

In *P. vivax* dosages of as little as 100 mg apparently render the gametocytes incapable of completing the sporogonous cycle, although they may persist in the blood for several days. These results confirm those of Mackerras and Ercole.[15] Little has been done on the extension of resistance to this drug to *P. vivax* gametocytes. However, since the development of resistance in the asexual parasites parallels that in *P. falciparum*, there is little probability that the gametocytes would differ in this respect.

3. *Pyrimethamine:* This is a drug of extreme importance in the control of malaria and, particularly, from the point of view of elimination of infectivity to mosquitoes. The sporontocidal action of this drug was early demonstrated by Foy and Kondi[16] and Shute and Maryon.[17] Subsequent reports from our laboratories have confirmed these results.[5, 9, 18] While resistance to pyrimethamine has been reported by many investigators from many situations in regard to the asexual stages of the various species of human malaria, much more significant from the standpoint of the present discussion have been the reports of extension of this resistance to the sexual forms. These studies have indicated that when resistant strains of *P. vivax* or *P. falciparum* are treated with pyrimethamine, the drug no longer exerts the sporontocidal effect characteristic of its action on non-resistant strains.[9, 19, 20] Further, the resistant character is readily transmitted through mosquitoes and persists through subsequent passages even in the absence of further contact with the drug. In these respects pyrimethamine is similar to chlorguanide. While pyrimethamine will undoubtedly continue to be a valuable drug for use in control and eradication programs, this unfortunate characteristic will undoubtedly seriously limit its usefulness in many areas at some time in the future.

4. *Primaquine:* Perhaps the most promising drug from the point of view of elimination of infectivity to mosquitoes is primaquine. Very little has been done concerning infectivity following primaquine therapy of *P. vivax*, but there is little

need for a more effective sporontocidal drug than chloroquine alone. The advantage of primaquine in the treatment of this species lies in its curative action. When used in combination with a good suppressive, as one of the 4-amino-quinolines, the hazard of relapse as well as immediate post-treatment infectivity can be eliminated in most cases.

In *P. falciparum* infections primaquine has the special advantage of rapidly removing viable gametocytes from the blood. In many cases it is not possible to infect mosquitoes even 24 hours after primaquine treatment; in a few infectivity may persist for as long as 3 or 4 days after treatment. This is somewhat in contrast to the persistence noted following chloroquine alone. Further, it appears that when used in combination with chloroquine in curative amounts, only small amounts of primaquine—as little as 30 to 90 mg in 1 to 3 days—are necessary to remove infective gametocytes.[8]

Recent work by Young[21] has shown a similar effect of even smaller doses of primaquine given daily, twice-weekly or weekly. Where dosages are as high as 15 to 30 mg single dose, even in the absence of a suppressive drug, the sporontocidal effect is almost immediate. Lower doses require a somewhat longer period.

This drug undoubtedly has great epidemiological significance. Even in the absence of specific diagnosis in the treatment of clinical attacks, or in the absence of case-finding blood surveys, the transmission rate could be decreased enormously in endemic areas by inclusion of primaquine in the routine treatment of clinical malaria. Toxicity of sporontocidal doses is apparently negligible, so that the drug can be used with little or no close observation of the patient. Future developments may even indicate use of the drug in mass prophylaxis, either in combination with a suppressive or alone, in an effort to eliminate transmission as an integral part of eradication programs.

It is apparent that each of the commonly used and most efficient antimalarial drugs has characteristics which in one way or another prevent it from being an ideal all-purpose drug. Nevertheless, with an understanding of these advantages and limitations of each drug, and of the normal patterns of the infection in man and its infectivity to mosquitoes, the use of drugs can be a valuable part of the programs of malaria eradication and control being undertaken throughout the world. I am even inclined to agree with those who feel that it is now and will become more and more an indispensable part of these programs.

REFERENCES

1. Jeffery, G. M., 1952. The infection of mosquitoes by *Plasmodium vivax* (Chesson strain) during the early primary parasitemias. *Am. J. Trop. Med. & Hyg.*, **1**: 612–617.
2. Jeffery, G. M., Young, M. D., Burgess, R. W., and Eyles, D. E., 1959. Early activity in sporozoite-induced *Plasmodium falciparum* infections. *Ann. Trop. Med. & Parasitol.*, **53**: 51–58.
3. Jeffery, G. M., and Eyles, D. E., 1955. Infectivity to mosquitoes of *Plasmodium falciparum* as related to gametocyte density and duration of infection. *Am. J. Trop. Med. & Hyg.*, **4**: 781–789.
4. Jeffery, G. M., and Eyles, D. E., 1954. The duration in the human host of infections with a Panama strain of *Plasmodium falciparum*. *Am. J. Trop. Med. & Hyg.*, **3**: 219–224.
5. Young, M. D., Hardman, N. F., Burgess, R. W., Frohne, W. C., and Sabrosky, C. W., 1948. The infectivity of native malarias in South Carolina to *Anopheles quadrimaculatus*. *Am. J. Trop. Med.*, **28**: 303–311.
6. Young, M. D., Eyles, D. E., and Burgess, R. W., 1948. Studies on imported malarias. 10. An evaluation of the foreign malarias introduced into the United States by returning troops. *J. Nat. Malaria Soc.*, **7**: 171–185.
7. Eyles, D. E., Young, M. D., and Burgess, R. W., 1948. Studies on imported malarias. 8. Infectivity to *Anopheles quadrimaculatus* of asymptomatic *Plasmodium vivax* parasitemia. *J. Nat. Malaria Soc.*, **7**: 125–133.
8. Jeffery, G. M., Young, M. D., and Eyles, D. E., 1956. The treatment of *Plasmodium falciparum* infection with chloroquine with a note on infectivity to mosquitoes in primaquine- and pyrimethamine-treated cases. *Am. J. Hyg.*, **64**: 1–11.
9. Jeffery, G. M., 1958. Infectivity to mosquitoes of *Plasmodium vivax* following treatment with chloroquine and other antimalarials. *Am. J. Trop. Med. & Hyg.*, **7**: 207–211.
10. Fairley, N. H., *et al.*, 1946. Researches on Paludrine (M.4888) in malaria. An experimental investigation undertaken by the L.H.Q. Medical Research Unit (A.I.F.), Cairns, Australia. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **40**: 105–153.
11. Shute, P. G., and Maryon, M., 1948. The gametocytocidal action of Paludrine upon infections of *Plasmodium falciparum*. *Parasitology*, **38**: 264–270.
12. Covell, G., Nicol, W. D., Shute, P. G., and Maryon, M., 1949. Studies on a West African strain of *Plasmodium falciparum*. II. The efficacy of Paludrine (Proguanil) as a therapeutic agent. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **42**: 465–476.
13. Ramakrishnan, S. P., Young, M. D., Jeffery, G. M., Burgess, R. W., and

McLENDON, S. B., 1952. The effect of single and multiple doses of Paludrine upon *Plasmodium falciparum*. *Am. J. Hyg.*, **55**: 239–245.

14. LIANG, A. B. G., 1956. Proguanil resistance-extension to the gametocytes of *Plasmodium falciparum*. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **50**: 496–504.
15. MACKERRAS, M. J., AND ERCOLE, Q. N., 1947. Observations on the action of Paludrine on malarial parasites. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **41**: 365–376.
16. FOY, H., AND KONDI, A., 1952. Effect of Daraprim on the gametocytes of *Plasmodium falciparum*. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **46**: 370.
17. SHUTE, P. G., AND MARYON, M., 1954. The effect of pyrimethamine (Daraprim) on the gametocytes and oocysts of *Plasmodium falciparum* and *Plasmodium vivax*. *Tr. Roy. Soc. Trop. Med. & Hyg.*, **48**: 50–63.
18. YOUNG, M. D., AND BURGESS, R. W., 1957. Effect of 25 milligrams of pyrimethamine on the infectivity of *Plasmodium vivax*, St. Elizabeth strain, to *Anopheles quadrimaculatus*. *Am. J. Trop. Med. & Hyg.*, **6**: 805–807.
19. YOUNG, M. D., AND BURGESS, R. W., 1959. Pyrimethamine resistance in *Plasmodium vivax* malaria. *Bull. World Health Organ.*, **20**: 27–36.
20. BURGESS, R. W., AND YOUNG, M. D., 1959. The development of pyrimethamine resistance by *Plasmodium falciparum*. *Bull. World Health Organ.*, **20**: 37–46.
21. YOUNG, M. D., 1959. The effect of small doses of primaquine upon malaria infections. *Indian J. Malariol.*, **13**: 69–74.

THE PHYSIOLOGY OF HIBERNATION AND ITS ROLE IN THE GEOGRAPHICAL DISTRIBUTION OF POPULATIONS OF THE *CULEX PIPIENS* COMPLEX

ASEFFA TEKLE

Laboratories of Medical Entomology, Department of Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland

In North America *Culex pipiens pipiens* Linn. was shown by Barr[1] to exist north of 39° N while *C. pipiens fatigans* Wied. is south of 36° N; collections between these latitudes were of one or the other form, or of intermediates. It would be of interest to know what prevents the extension of the range of *pipiens* to the south and of *fatigans* to the north. Farid[2] showed that there was no significant difference in the reaction of immature stages of *pipiens* and *fatigans* to different temperatures. MacMillan[3] found that the Lawrence, Kansas, population of the *C. pipiens* complex was typical *pipiens* early in the year, but became variable and more *fatigans*-like later in the season. This author suggested that only the individuals similar to *pipiens* were able to overwinter successfully, while the increase of *fatigans* in the late season could be due to influx from the south.

It was the purpose of this investigation to determine what effect, if any, temperature might have as a limiting factor in the distribution of the *C. pipiens* complex, and to reveal any differences that might exist in the adaptations of the two subspecies to hibernation.

MATERIALS AND METHODS

The *C. p. pipiens* strain originated from material collected on the outskirts of Baltimore; the *C. p. fatigans* was a Philippine strain.[4] Throughout the study both subspecies were reared under comparable conditions with respect to concentration of larvae, temperature, and availability of food.

In the longevity experiments, mosquitoes, whose rearing and post emergence histories were known, were aspirated, counted and introduced into holding chambers. These chambers were then placed in a special temperature-control cabinet having combined refrigeration and heating units which maintained the temperature at 5°C. The mosquitoes undergoing simulated hibernation were kept in gauze-covered lantern chimneys. Each chimney was inserted in a half-pint wax paper ice-cream carton. A thick pad of cellucotton was kept moistened on the top of the chimney so as to maintain a high humidity and to allow the mosquitoes access to water.

For fat extraction mosquitoes were killed by placing them at −10°C, after which they were carefully separated and dried to a constant weight in an oven at 70 to 80°C. They were then extracted with ether in a Soxhlet apparatus for 5 hours, and dried again to a constant weight. Each extraction sample consisted of 5 mosquitoes. The difference in weights before and after extraction was taken as the weight of the ether-soluble components, which were considered to be lipids. Determination of the weights was accomplished by using a torsion balance with sensitivity of 5 μg. To insure that no part of the mosquito would be broken, the specimens were gently tapped out of the thimble while the thimble was still moist.

Oxygen consumption determinations were carried out with slightly modified Warburg respirometers using flasks of approximately 20 ml volume. The center well contained 0.2 ml of 20% KOH. To provide a large absorption surface, the center well was lined with rectangularly cut filter paper, and its top was covered with properly fitting wire gauze to prevent mosquitoes from dropping into the KOH. The desired number of mosquitoes was drawn into an aspirator and was then introduced into the main compartment of the Warburg flask. This was achieved by first sealing all the manometers and vessels with petrolatum except one side arm of each vessel. This side arm was left open and was free of grease. The mouth of the aspirator was then brought to the top of this unsealed side-arm opening and the mosquitoes were gently allowed to move into the side arm and from here into the main compartment; some also moved into the other side arm. The manometers were then placed in a constant temperature water bath.

TABLE 1

Effect of temperature on developmental time and on percentage of adult emergence

Rearing Temperature (°C.)	No. replicates*	Days for development egg to adult†		% of adult emergence	
		fatigans	*pipiens*	*fatigans*	*pipiens*
35	4	8 ± 0.48‡	—	29 ± 0.7‡	0
30	4	11 ± 0.46	12 ± 0.24	70 ± 0.92	65 ± 0.56
25	4	13 ± 0.43	14 ± 0.72	90 ± 1.32	91 ± 0.93
20	4	22 ± 0.48	26 ± 1.45	86 ± 1.11	85 ± 1.3
15	4	41 ± 0.97	48 ± 1.94	86 ± 0.6	83 ± 0.7
10	2	60 ± 3.1	64 ± 3.8	5 ± 2.6	32 ± 3.1

* In each replicate there were 2 pans of 100 larvae for each subspecies.
† Number of days required for 50% of the specimens.
‡ Mean ± standard error.

Temperature below that of the laboratory was obtained by placing the water bath in a cold room at 2–3°C. Usually a large amount of ice cubes was necessary to bring the water bath near the desired temperature quickly. After the desired temperature was achieved, temperature equilibration and subsequent readings were made at intervals of 15 minutes for 2 hours. No agitation of the water bath was needed since the organisms were not in a liquid medium; furthermore, basal metabolism was best studied if the mosquitoes were not disturbed. The flask volumes were corrected for different temperatures using the method of Umbreit *et al.*[5] The change in the level of the manometer arm was read as microliters of oxygen consumed during a given period of time.

EXPERIMENTAL RESULTS

Experiment I. The purpose of this experiment was to determine whether there is a difference in temperature tolerance between *C. p. fatigans* and *C. p. pipiens.*

About 30 fertile egg rafts were taken from the *fatigans* colony and were divided into 6 lots, each comprising 4 to 5 egg rafts. Each lot of egg rafts was then placed in a bowl of water and kept at 10, 15, 20, 25 or 30°C until hatching was complete. Immediately thereafter, 2 groups of 100 larvae were transferred from each bowl into enamel pans full of water. The larvae from a given lot were reared at the same temperature as that at which they had hatched. The same procedure was simultaneously applied to *pipiens.* Thus in a given temperature-controlled cabinet there were 4 pans, 2 of *pipiens* and 2 of *fatigans,* each with 100 larvae.

In Table 1 the egg-to-adult period is presented as the number of days required for emergence of 50% of the adults from the rearing pans. The effect of low or high temperature on the life cycle of *fatigans* and *pipiens* was to increase or decrease the developmental times within certain limits. The extreme range for normal development in *fatigans* was 35 to 10°C, whereas for *pipiens* it was 30 to 10°C. The optimum temperature for adult emergence of both *fatigans* and *pipiens* was 25°C; any temperature above or below this gave a progressively lower percentage of adult emergence. *Fatigans* at any temperature started to pupate and emerge 2 to 3 days earlier than did *pipiens.* At 10°C, 32% of the *pipiens* but only 5% of the *fatigans* larvae were able to reach the adult stage. At 35°C no *pipiens* larvae was able to pupate, whereas 29% of the *fatigans* larvae pupated and emerged as adults.

Eggs will hatch at temperatures which are too low or too high to permit survival of the larva which hatched from them. Adult female *pipiens* were given blood, and after 4 to 5 days were placed in a refrigerator at 5°C. These females laid eggs which hatched 8 to 10 days later, but all larvae died within a few hours. *Pipiens* larvae hatching at 35°C were able to live only to the second instar. However, if *pipiens* larvae which hatched at room temperature (22 to 25°C) were permitted to grow to the late first or early second instar, they could then be transferred to 35°C and successfully complete their development to the adult stage.

From the results of this experiment, it is con-

cluded that for *pipiens* and *fatigans* the optimum temperature is around 25°C, whereas *pipiens* larvae are better adapted for growth at a lower temperature than are those of *fatigans*, and *fatigans* larvae are able to develop at higher temperatures than are those of *pipiens*.

Experiment II. The objective in this experiment was to study the effect of temperature on the body weights and fat contents of female *C. p. fatigans* and *C. p. pipiens*. A gradual fall in temperature with the approach of winter might cause a change in the body size and composition of the mosquitoes, resulting in a greater accumulation of nutritive reserves for hibernation.

Collections of 20 to 30 fertile egg rafts, taken separately from the *fatigans* and *pipiens* colonies, were divided into 3 lots, each placed in a bowl of water and kept at 30, 25 and 15°C until hatching was complete. Immediately thereafter, 2 groups of 100 larvae were counted from each bowl and transferred to enamel pans full of water. The larvae from a given lot were reared at the same temperature as that at which they hatched. Adult females reared from these pans were killed, stored, weighed, and analyzed for fat as described above. These adults were 1 day old, and unfed, at the time they were sacrificed. Determinations of weight and fat content were made with groups of 5 individuals.

Table 2 shows that the lower rearing temperatures produced larger adult mosquitoes with greater fat content. The body weights for 5 adult female *fatigans* were 3.394 mg at 30°C and 4.782 mg at 15°C, an increase of more than 1 mg; those of *pipiens* females at these temperatures were 3.511 and 5.019, respectively. However, when the increase of fat content was divided by the body weight, the ratio remained constant. Thus, although the body weight and fat content in the mosquitoes reared at the low temperature were significantly higher than in those reared at the higher temperature, the relative amount of fat accumulated at different rearing temperatures remained constant.

In nature, in early winter, hibernating female mosquitoes are usually found with large, fully developed fat bodies, which are gradually exhausted throughout the winter. This was true of wild *pipiens* collected from a cave near Baltimore on December 19, 1957. Four determinations were made, each with a sample of 5 specimens, with the following results: dry weight, 5.875 ± 0.34 mg; fat, 2.325 ± 0.92 mg; proportion of fat, 46.8 ± 1.2%.

To determine the effect of carbohydrates on the fat reserve, two groups of mosquitoes, reared and kept at 25°C and 15°C, were given 15% sugar solution for 2 weeks. These, as shown in Table 3, not only contained a greater amount of fat, but also the ratio of fat to body weight was higher than that in the corresponding unfed mosquitoes. Comparison of Tables 2 and 3 shows that the proportion of fat in the sugar-fed groups was 29.8 to 37.2%, while in the unfed mosquitoes it was approximately 20%. Thus the fed adults were more nearly like the wild caught specimens.

Another measure of the change of body size was the wing length; it was observed that the lower the rearing temperature of the larvae, the longer were the wings of the emerging adults (table 4).

TABLE 2

Dry weight and fat content of unfed adult females reared at 30, 25, and 15°C

Subspecies	Rearing temp. (°C)	Mg per sample of 5 females*		
		Dry wt.	Fat	% Fat*
fatigans	30	3.394 ± 0.09	0.665 ± 0.04	19.5 ± 0.5
	25	4.324 ± 0.12	0.942 ± 0.05	20.6 ± 0.4
	15	4.782 ± 0.13	1.052 ± 0.05	21.8 ± 1.0
pipiens	30	3.511 ± 0.11	0.748 ± 0.03	20.0 ± 0.8
	25	4.494 ± 0.11	1.093 ± 0.05	22.1 ± 0.6
	15	5.019 ± 0.08	1.150 ± 0.04	22.1 ± 0.7

* Mean ± standard error. Each value is based upon 12 replicate determinations, with 5 specimens per sample.

TABLE 3

Dry weight and fat content of adult females reared at 25 and 15°C and fed for 2 weeks on 15% sugar solution

Subspecies	Rearing temp. (°C)	Mg per sample of 5 specimens*		
		Dry wt.	Fat	% Fat*
fatigans	25	4.846 ± 0.51	1.508 ± 0.07	29.8 ± 2
	15	5.263 ± 0.1	1.930 ± 0.1	34.9 ± 0.6
pipiens	25	4.943 ± 0.21	1.610 ± 0.08	32.8 ± 1.5
	15	5.613 ± 0.14	2.09 ± 0.12	37.2 ± 1.1

* Mean ± standard error. Each value is based upon 12 replicate determinations with 5 specimens per sample.

TABLE 4

Changes in wing length with rearing temperatures

Rearing temperature (°C)	Wing length in mm*	
	fatigans	*pipiens*
30	4.77 ± 0.01	4.79 ± 0.02
25	4.82 ± 0.03	4.83 ± 0.02
15	4.94 ± 0.02	5.02 ± 0.01

* Mean (per group of 80 mosquitoes) ± standard error.

Experiment III. The objective in this experiment was to determine the effect of larval rearing temperatures and prehibernation adult feeding on the longevity of the adults during hibernation. Groups of *C. p. pipiens* and *C. p. fatigans* larvae were reared at 25° and 15°C, and the emerging adults were placed in cages at 5°C where they were observed for longevity. The adults were either unfed or fed as described below. The results are presented in Table 5. The observations were continued until 80% of the females had died; this period was referred to as the 20% survival time. There were 2 to 4 replicates of each observation; in each replicate there were 20 to 40 females.

Recently emerged, unfed *pipiens* and *fatigans* females survived for only about 2 weeks.

Adult females reared as larvae at 25° and 15°C, and maintained at these temperatures for 2 weeks on 15% sugar solution, were much better prepared for hibernation than were unfed females. The mean 20% survival times for *fatigans* and *pipiens* were 51 and 85 days, respectively, in the 15°C groups. Although in both *pipiens* and *fatigans* the groups reared and kept at 15°C lived longer than groups reared at 25°C, the differences were not significant. In both the fed and unfed groups, *pipiens* outlived *fatigans* at 5°C.

Females of *pipiens* and *fatigans* reared as larvae at 25°C were kept at this temperature and

TABLE 5

Longevity of mosquitoes at 5°C

Rearing temp. (°C)	Condition of mosquitoes	No. of replicates*	Time in days for 20% survival†	
			fatigans	*pipiens*
25	Young and unfed	4	12 ± 1.70	14 ± 1.94
15	Young and unfed	3	14 ± 3.06	15 ± 3.36
25	2 weeks on sugar soln.	4	47 ± 2.66	80 ± 3.64
15	2 weeks on sugar soln.	3	51 ± 3.06	85 ± 7.15
25	2 weeks on sugar and blood	3	33 ± 1.70	68 ± 1.7
25	Only blood	2	8 ± 3.78	10 ± 3.28
25	Immediate post-oviposition refrigeration	3	5 ± 0.68	5 ± 0.68
25	Sugar-fed after oviposition	2	22 ± 3.78	33 ± 3.14

* 20 to 40 females per replicate.

† Mean ± standard error.

fed on sugar solution for 2 weeks, and then allowed to take blood from chickens. Within 8 hours after the blood meal, each group was placed in a refrigerator at 5°C. The mean number of days for 20% survival of *pipiens* was 68, while for *fatigans* it was 33. These surviving *fatigans* were observed to determine the time required for 100% mortality; this was found to be 50 to 52 days.

To determine whether blood alone is sufficient for prehibernation conditioning, the mosquitoes were reared at 25°C and kept in a cage for 3 to 4 days without food. On the 5th day, they were permitted to feed on a chicken and, within 8 hours, the blood-fed mosquitoes were placed in a 5°C refrigerator. Both *fatigans* and *pipiens* lived only 8 to 10 days.

In order to determine whether old mosquitoes, that is, mosquitoes which had had a blood meal and had laid eggs, could also go into hibernation, *pipiens* and *fatigans* females were kept in a 25°C incubator and fed 15% sugar solution. The mosquitoes were kept for 1 week, then given a meal of chicken blood. The blood-fed females were transferred to a lantern chimney which covered a dish containing water. Here they were left until they laid their eggs; then they were divided into 2 groups. One group was immediately placed in the 5°C refrigerator, while the other was transferred to another lantern chimney, which was placed over a Petri dish with a cotton pad soaked with 15% sugar solution. These mosquitoes were allowed to feed on sugar solution for 5 days; then they also were placed at 5°C refrigeration for hibernation tests.

Table 5 shows that both *pipiens* and *fatigans* which were subjected to 5°C immediately after oviposition, lived only about 5 days. Both *pipiens* and *fatigans* females survived for longer periods when given a sugar meal after egg laying; but, again, *pipiens* outlived *fatigans*.

The results of this experiment indicate that the mosquitoes best conditioned for hibernation are those which, after emergence, have fed on sugar solution or a combination of sugar solution and blood. Regardless of the rearing conditions unfed emerging adult mosquitoes do not live more than a few days and so cannot go into hibernation. Similarly, females which have fed only on blood, or those which have oviposited and have not had a chance to feed again, do not live more than a few days and so cannot go into hibernation. However, mosquitoes which had sugar solution after oviposition lived for a considerable period of time, although not as long as the young and sugar-fed adult mosquitoes. In general, sugar-fed *pipiens* lived much longer than sugar-fed *fatigans*, but there was no significant difference between the unfed *pipiens* and *fatigans*.

Experiment IV. The objective in this experiment was to measure the oxygen consumption of young and unfed adult female *C. p. fatigans* and *C. p. pipiens* at 25, 15 and 7°C, and also to determine whether the temperature at which the larvae were reared would later affect the rate of metabolism of the adults. The mosquitoes were reared and kept at 25°C and 15°C. The adult females were tested 8 to 10 hours after emergence. The rate of oxygen consumption was measured by means of a Warburg apparatus. The number of mosquitoes present in each vessel varied according to the temperature at which the experiment was performed. Thus, at 25°C only 10 mosquitoes in each vessel were required to give a satisfactory rate of change in the manometer arm, while at 15°C and 7°C, 25 mosquitoes were required to get a proper rate of change. This was true for both *pipiens* and *fatigans*. The movement of the mosquitoes at all temperatures during the experiments was slight, and so it is believed that the higher oxygen consumption observed in all of the mosquitoes at higher temperature was not due to an increase in muscular activity.

The oxygen consumption is recorded in Table 6 as microliters of oxygen consumed per hour per mg body weight. In both *fatigans* and *pipiens*, reared at 25°C, the higher the assay temperature the greater was the oxygen consumption. The decrease in oxygen consumption when the temperature was dropped from 25° to 15°C was about

TABLE 6

Oxygen consumption of unfed mosquitoes

Rearing temp. (°C)	Assay temp. (°C)	No. of replicates	Microliters of oxygen/hr./mg*	
			fatigans	*pipiens*
25	25	4	4.57 ± 0.39	5.86 ± 0.52
	15	4	2.83 ± 0.19	3.06 ± 0.15
	7	4	1.01 ± 0.10	1.22 ± 0.20
15	25	4	6.32 ± 0.10	6.94 ± 0.08
	15	4	2.55 ± 0.13	2.62 ± 0.13
	7	4	1.04 ± 0.01	1.23 ± 0.01

* Mean ± standard error.

TABLE 7

Oxygen consumption of mosquitoes fed on sugar for 2 weeks

Rearing temp. (°C)	Assay temp. (°C)	No. of replicates	Microliters of oxygen/hr./mg*	
			fatigans	*pipiens*
25	25	4	10.48 ± 0.56*	18.03 ± 0.27
	15	4	6.51 ± 1.10	13.36 ± 0.42
	7	4	2.99 ± 0.21	6.47 ± 0.53
15	25	2	11.8 ± 1.70	19.66 ± 0.46
	15	2	7.46 ± 1.70	14.60 ± 0.58
	7	2	3.16 ± 0.25	7.21 ± 0.95

* Mean ± standard error.

1.5 to 2 times, whereas when dropped from 15° to 7°C, which is less than 10°, it was about 2.5 times in both groups. For a given assay temperature the oxygen consumption of *pipiens* was always slightly higher than that of *fatigans*, although the difference was not significant.

The response of some insects to temperature may be influenced by their exposure to temperature at an earlier stage in their development. Table 6 shows that although some mosquitoes reared at 15°C apparently had slightly higher rates of oxygen consumption than those reared and kept at 25°C, the differences were not significant. Again it is seen that for a given temperature, the oxygen consumption of *pipiens* was slightly higher than that of *fatigans*, and that the difference was greater at higher assay temperatures.

Experiment V. Since it was found that a prehibernation meal of sugar solution was essential for survival, and that sugar-fed females of *pipiens* outlived those of *fatigans*, the objective in this experiment was to determine whether there is a difference in the rate of oxygen consumption between sugar-fed *fatigans* and sugar-fed *pipiens*.

A number of *fatigans* and *pipiens* female adults, reared and kept at 25°C and treated identically, were maintained on 15% sugar solution for 2 weeks. The mosquitoes were starved for 8 to 10 hours prior to the oxygen consumption determinations. It is evident from Table 7 that 2 weeks of sugar feeding increased the oxygen consumption of mosquitoes to approximately 2 to 3 times that of unfed mosquitoes. These mosquitoes also responded to changes in assay temperatures. Of special interest were the differences in oxygen consumption between sugar-fed *fatigans* and sugar-fed *pipiens*. Thus, at a given temperature a sugar-fed *pipiens* consumed oxygen about twice as fast as sugar-fed *fatigans*; this difference was more marked at lower temperatures. The results obtained with mosquitoes reared and kept at 15°C and maintained on 15% sugar solution for 2 weeks were essentially the same as those of the 25°C groups.

Experiment VI. The females of *pipiens*, when fed on sugar solution, lived longer than did those of *fatigans* under simulated hibernation; and the former showed a greater rate of respiration. The purpose of this experiment was to compare the utilization of fat reserves in the two forms during the course of hibernation. *Pipiens* and *fatigans* reared identically under optimum conditions (100 larvae per pan, adequate amounts of food, and at 25°C) were kept at room temperature for 2 weeks on 15% sugar solution. Three lots of each subspecies, each lot comprised of 100 females in a lamp chimney, were placed in a refrigerator at 5°C. After 2, 4, and 6 weeks, samples of mosquitoes were removed for fat extraction. On the day the mosquitoes were placed in the refrigerator, a zero time fat extraction was also made.

The results presented in Table 8 show that as time advanced the amount of fat diminished. However, the rate of fat utilization in *pipiens* was 1.5 to 2 times greater than that in *fatigans*. At the end of the 6th week all but a few of the *fatigans* were dead, while a number of the *pipiens* were alive; the average fat content per 5 female *fatigans* was 1.008 mg while it was 0.605 mg per 5 *pipiens*. The fact that *fatigans* females began to die at a time when they still contained almost twice as much fat as the surviving *pipiens* suggests that the latter have a superior mechanism for mobilization of fat reserves during the hibernation period.

Experiment VII. The objective in this experiment was to determine the direct effect of temperature on egg production. *C. p. pipiens* and *C. p. fatigans*, reared and kept at 25°C and maintained on 15% sugar solution, were given a blood meal at room temperature and immediately placed in a series of lamp chimneys. Paired lamp chimneys, each with an equal number of *pipiens* or *fatigans*, were placed at 25, 20, 15, 10 and 5°C in a previously adjusted incubator or refrigerator. The time taken to digest blood and to lay eggs, and the number of egg rafts laid at each temperature are recorded in Table 9.

TABLE 8

Body fat changes during simulated hibernation of mosquitoes

Subspecies	No. of mosq.*	Weeks at 5°C.	Mg weight per 5 specimens			
			Dry wt.†	Fat cont.†	Decrease in fat	% Fat
fatigans	30	0	4.809 ± 0.13	1.623 ± 0.06	—	33.7
	30	2	4.208 ± 0.08	1.375 ± 0.06	0.248	32.6
	30	4	3.770 ± 0.12	1.180 ± 0.03	0.195	31.2
	30	6	3.370 ± 0.08	1.008 ± 0.11	0.172	29.9
pipiens	30	0	4.919 ± 0.08	1.721 ± 0.09	—	35.0
	30	2	3.953 ± 0.17	1.338 ± 0.07	0.383	33.8
	30	4	3.337 ± 0.09	0.977 ± 0.08	0.361	29.3
	30	6	2.855 ± 0.14	0.605 ± 0.08	0.372	21.2

* Reared and kept at 25°C, and maintained on sugar solution for 2 weeks.
† Mean ± standard error.

TABLE 9

Effect of different temperatures on egg production

Subspecies	No. of replicates	Temp. (°C)	Blood digestion time (days)	Oviposition time (days)	% of females ovipositing
fatigans	2*	25	2–3	4–5	100
	2	20	4–5	7–10	100
	2	15	7–10	17–21	62.7
	2	10	18–22	35–40	13.2
	2	5	—	—	—
pipiens	2	25	2–3	4–5	100
	2	20	5–6	7–10	100
	2	15	7–10	15–20	70
	2	10	15–20	30–35	23.3
	2	5	40–50	>60	—

* Each replicate contained 30 mosquitoes.

It is obvious that the lower the temperature at which the blood-fed mosquitoes were kept, the longer the time for digestion and egg laying, and the fewer the number of egg rafts laid. All of the mosquitoes kept at 25°C oviposited, at 10°C only 13 and 23% did so, and at 5°C no eggs were produced. When the mosquitoes that did not lay eggs at 15°C and 10°C were dissected, they showed completely developed eggs. The *fatigans* females kept at 5°C did not live long enough to digest their blood meals. The condition of their ovaries was not observed. The 5°C *pipiens* females digested their blood meals after 40 to 50 days, but had not oviposited after 60 days.

Experiment VIII. Hibernating mosquitoes may be found with ovaries varying in their degree of development, but they seldom are mature. This experiment was designed to demonstrate a mechanism whereby some hibernating mosquitoes may have partially developed ovaries. Since it is *pipiens* that hibernates in nature, and since *pipiens* is the form that lasts long enough to digest the blood meal at 5°C, the study was limited to this subspecies.

Females which had been reared and kept at 25°C and fed on 15% sugar solution were allowed to engorge on a chicken. They were then divided into 5 lots, 4 of which were comprised of 20 females each, while the 5th lot was comprised of 40 females. Each lot, after being kept at room temperature for a designated period of time, was subjected to 5°C refrigeration. After the refrigeration period (which was the time needed for complete blood digestion), some of the mosquitoes were dissected immediately, while the rest were placed at room temperature for 6 days and then dissected. The results are given in Table 10.

Lot 1 was kept for 96 hours at room temperature after blood feeding and then placed at 5°C. Six egg rafts were laid within 48 hours and all mosquitoes died 5 days later. The eggs were left at that temperature and all hatched within 10 days, but all of the larvae died immediately.

Lot 2 deposited no eggs, but 10 of the females dissected after 10 days of refrigeration showed fully developed ovarian follicles (Cristophers' stage V). The 10 remaining mosquitoes were brought back to room temperature for 6 days; they deposited 8 egg rafts.

At the end of the 20 day refrigeration period,

TABLE 10

The effect of 25°C pre-incubation time on ovarian development at 5°C

Lot. no.	No. of blooded mosquitoes	25°C pre-incubation time (hours)	Blood digestion time at 5°C (days)	Ovarian stages* after reincubation at 25°C	
				0 day	6 days
1	20	96	0	V	—
2	20	48	10	V	V
3	20	16	20	V	V
4	20	8	30	III	IV
5	40	2	50	II	II†

* Christophers' classification.

† 3 to 4 follicles reached stage V.

only 15 females of Lot 3 were alive; 8 which were dissected immediately possessed stage V ovaries. The remaining 7 mosquitoes were brought to room temperature and observed for 6 days, during which period 4 egg rafts were laid.

At the end of 30 days of refrigeration, 6 of 14 surviving Lot 4 females had partially developed ovarian follicles (Christophers' stage III). The remaining 8 mosquitoes were brought to room temperature for 6 days; the follicles reached Christophers' stage IV, but no oviposition took place.

In Lot 5, 22 females survived the 50 day refrigeration period, during which time the entire blood meal had been digested. Ten of these were dissected and the ovarian follicles were found to be in Christophers' stage II. The 12 remaining mosquitoes were brought to room temperature and observed for 6 days. Since no egg rafts were found by the 7th day, a chicken was made available to the females, and, the next day, 8 mosquitoes were found to be fully engorged. All of them laid viable eggs. The 4 unfed mosquitoes were dissected and only 3 to 4 follicles were found to be in Christophers' stage V, while the remainder were in stage II.

Thus it is evident that lower temperatures not only lengthen the time required for digestion of blood and for oviposition, but also inhibit oviposition. At 5°C there was no oviposition and the ovarian follicles did not develop beyond Christophers' stage II, even when all the blood had been digested. A drop in temperature induced a cessation or retardation of the ovaries' growth that might have started at a favorable temperature. Thus, if a mosquito were blood-fed, the ovary would continue to develop until subjected to a low temperature, at which time ovarian growth would stop, or proceed at most at a very low rate.

It is worth-while to note that in Lot 5 female mosquitoes were subjected to 5°C for 50 to 60 days, and when brought to room temperature and given a blood meal, they laid eggs which hatched. This shows that the sperm were viable at the cold temperature at least for 50 to 60 days.

DISCUSSION

The effect of low temperatures of the breeding water was to lengthen the time required for egg-to-adult development, and to increase the wing length, body weight and fat content of *C. p. pipiens* and *C. p. fatigans* females. The ratio of fat content to dry body weight, however, remained more or less constant at any breeding temperature. It seems logical, therefore, to consider that the breeding temperature is not an essential factor for conditioning the females for hibernation.

Buxton[6] observed that *C. pipiens* females contain a great accumulation of fat in the fall and exhaust approximately 85% of it throughout the winter. As seen in Table 3, the greatest accumulation of fat in both *pipiens* and *fatigans* occurred when the females were allowed to feed for a certain period of time on a sugar solution. These results agree with the observations of Roubaud,[7] Tate and Vincent,[8] and Wesenberg-Lund[9] that hibernating females of *pipiens* do not require a blood meal for fat body formation. Tate and Vincent showed also that, whereas the unfed females lived an average of 11 days at 16 to 20°C, the females fed on apple for 5 weeks stored up sufficient reserve to allow about 15% of them to survive over 4 months of starvation. This is long enough to carry them through the winter months. In nature, mosquitoes emerging from their breeding waters may wander for a while in search of some energy-supplying food before entering a hibernation site. In southern California, Reeves *et al.*[10] found that about 98% of hibernating *Culex tarsalis* had no trace of blood in their guts nor any sign of ovarian development; thus most of these mosquitoes must have obtained their energy and fat building materials from plant juices.

In the study of longevity it was noted that females of *C. p. pipiens* survived longer than did

those of *C. p. fatigans* when both were kept at 5°C. This suggested that *pipiens* has some physiological mechanism whereby it can be relatively independent of temperature, within limits, in nature. Like other poikilothermic organisms, mosquitoes vary their metabolism with fluctuations in the environmental temperature. At any temperature the young and unfed adults consumed less oxygen than did old and sugar-fed adults, and old and sugar-fed *pipiens* consumed more oxygen than did old and sugar-fed *fatigans* of equal weight. This was interpreted as a metabolic compensation to temperature on the part of *pipiens*, which normally inhabits colder regions. It has been generally assumed that lowering of temperature decreases metabolic activity in poikilotherms, while raising the temperature increases such activity. However, evidence has accumulated of a homeostatic tendency in many rate functions. Bullock[11] and Prosser[12] have reviewed the available data relevant to metabolic compensation in relation to temperature. Prosser[12] states: "The rate of oxygen consumption by cold-acclimated poikilothermic animals is usually higher than for warm-acclimated ones, whether the acclimation is seasonal . . . or latitudinal . . . or altitudinal."

In female *pipiens* and *fatigans* the stored fat decreased during the course of simulated hibernation, but females of *fatigans* did not utilize their fat reserves at the same rate as did *pipiens*, and as a result, *pipiens* lived longer than did *fatigens*. In fact, when the *fatigans* females died, they still had a large amount of fat reserve. The respective rates of fat utilization by these mosquitoes were comparable to their rates of oxygen consumption. This is additional evidence that females of *pipiens* can utilize their fat reserve, while those of *fatigans* can not, and as a result, the former survive the winter while the latter die.

The ability of *pipiens* females to utilize their fat reserve at low temperatures, and the lack of this ability in *fatigans*, may be an important reason for the geographic separation of the two subspecies. The latter may be unable to establish itself in northern regions or at high altitudes because of its inability to survive hibernation at low temperature. This could be a barrier to the northern extension of the *fatigans* range. However, it is not yet understood why *pipiens* is not able to inhabit southern regions. Farid[2] has suggested that *pipiens* may be unable to mate successfully at elevated temperatures.

In the case of mosquito species that hibernate in the adult stage, it is commonly thought that prehibernation blood meals are used for the formation of a large reserve of fat instead of for the development of eggs. This phenomenon, known as gonotrophic dissociation, was first reported by Swellengrebel.[14] Inasmuch as blood feeding and the maturation of the ovaries seem to be complementary processes at room temperature, the accumulation of fat while the ovaries remain undeveloped would appear to be the result of a change in the metabolism of the mosquitoes due to either internal or external stimulation. The present studies show that in *C. pipiens*, blood alone is not an adequate prehibernation meal, and that low temperatures have the effect of inhibiting further ovarian development in those mosquitoes which may have fed and rested for a while in a warmer environment. Thus partially developed ovaries may be found occasionally in hibernating mosquitoes.

SUMMARY

Culex pipiens pipiens and *C. p. fatigans* were subjected to a comparative study on the role of temperature in growth, longevity, respiration, fat accumulation and utilization, and egg production. In both subspecies, the lower the temperature the longer was the developmental time, the heavier was the body weight, the greater was the amount of fat, and the longer was the wing length. However, the proportion of fat in the mosquitoes reared at all temperatures remained constant. In both subspecies sugar-fed mosquitoes stored a large amount of fat, although this was not as great as that observed in females caught in nature during winter. The lower the temperature the lower was the rate of oxygen consumption. The sugar-fed mosquitoes consumed oxygen at a higher rate than did young and unfed mosquitoes. Young and unfed mosquitoes of both subspecies had the same rate of oxygen consumption; but following sugar feeding, this rate was significantly higher in *pipiens* than in *fatigans*. The rearing temperature had no effect on the rate of oxygen consumption of the adult mosquitoes. The dry body weight of previously sugar-fed *pipiens* and *fatigans* decreased during simulated hibernation; however, the rate of fat decrease in *pipiens* was greater than in *fatigans*. At the time when *fatigans* females were dying, their fat content was almost twice that of *pipiens*; at this time the surviving *pipiens* females outnumbered

fatigans females by 3 to 1. As temperature decreased, the time required for blood digestion and for oviposition increased, and fewer females oviposited. At 5°C *pipiens* females digested their blood meals in about 60 days but no oviposition took place. On the other hand, *fatigans* did not survive long enough to digest their blood meals.

From the above experimental results it was concluded that (1) the larval rearing temperature has no effect on the ability of the emerging mosquitoes to go into hibernation directly. (2) Prior to overwintering, adult female mosquitoes require carbohydrates, or a combination of carbohydrates and a blood meal to carry them through the winter. (3) Females of southern *fatigans*, like those of northern *pipiens*, are capable of accumulating fat reserves if given a perhibernation carbohydrate diet. (4) Hibernating *pipiens* females outlive *fatigans* females because they are able to utilize their fat reserves, whereas *fatigans* can not do so. (5) The inability of *fatigans* females to survive long hibernation periods at low temperatures may be an important reason for the absence of this subspecies from colder regions.

ACKNOWLEDGMENTS

The author is indebted to Dr. Lloyd E. Rozeboom for suggestions and criticisms. This work was supported in part by a grant from the National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

REFERENCES

1. Barr, A. R., 1957. The distribution of *Culex p. pipiens* and *C. p. quinquefasciatus* in North American. *Am. J. Trop. Med. & Hyg.*, **6**: 153–165.
2. Farid, M. A., 1949. Relationships between certain populations of *Culex pipiens* Linnaeus and *Culex quinquefasciatus* Say in the United States. *Am. J. Hyg.*, **49**: 83–100.
3. McMillan, M. L., 1958. Study of a naturally occurring population intermediate between *C. p. pipiens* and *C. p. quinquefasciatus*. *Am. J. Trop. Med. & Hyg.*, **7**: 505–511.
4. Rozeboom, L. E., 1958. Hybridization of *Culex pipiens fatigans* Wiedemann from the Philippine Islands with American strains of *Culex pipiens* group. *Am. J. Trop. Med. & Hyg.*, **7**: 526–530.
5. Umbreit, W. W., Burris, P. H., and Stauffer, J. F., 1957. *Manometric Techniques*. Burgess Publishing Co., Minneapolis.
6. Buxton, P. A., 1935. Changes in the composition of adult *Culex pipiens* during hibernation. *Parasitology*, **27**: 263–265.
7. Roubaud, E., 1933. Essai synthetique sur la vie du moustique commun *Culex pipiens*. L'evolution humaine et les adaptations biologique du moustique. *Ann. sc. nat.*, **16**: 5–168.
8. Tate, P., and Vincent, M., 1936. The biology of autogenous and anautogenous races of *Culex pipiens* L. *Parasitology*, **28**: 115–145.
9. Wesenberg-Lund, C., 1920–21. Contributions to the biology of the Danish Culicidae. *Mem. Acad. Roy. Sc. Lett. Danemark*, sect. sc., 8me ser. 7: 210.
10. Reeves, W. C., Bellamy, R. E., and Scrivani, R. P., 1958. Relationship of mosquito vectors to winter survival of encephalitis viruses. I. Under natural conditions. *Am. J. Hyg.*, **67**: 78–89.
11. Bullock, T. H., 1955. Compensation for temperature in the metabolism and activity of poikilotherms. *Biol. Rev.*, **30**: 311–342.
12. Prosser, C. L., 1955. Physiological variation in animals. *Biol. Rev.*, **30**: 229–262.
13. Prosser, C. L., 1957. The species problem from the viewpoint of a physiologist. In *The Species Problem*, pp. 339–369. American Association for the Advancement of Science Publication No. 50.
14. Swellengrebel, N. H., 1929. La dissociation des fonctions sexuelles et nutritives (dissociation gonotrophique) d'*Anopheles maculipennis* comme cause du paludisme dans les pays-bas et ses rapports avec "l'infection domiciliaire". *Ann. Inst. Pasteur*, **43**: 1370–1389.

THE EFFECT OF TEMPERATURE AND ILLUMINATION ON MATING OF *CULEX PIPIENS PIPIENS* L. AND *C. P. FATIGANS* WIED.*

ANNE C. M. PARKER AND LLOYD E. ROZEBOOM

Laboratories of Medical Entomology, Department of Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland

The reasons for the northern and southern distributions, respectively of *Culex pipiens pipiens* and *C. p. fatigans* are not clearly understood. These two populations are now generally accepted as representing subspecies, inasmuch as specimens and populations occur in nature in zones of overlapping ranges which are similar to experimentally produced hybrids.[1-4] Although we have not been able to detect a definite barrier between North American populations,[5] or even between these and *fatigans* from the Philippine Islands,[6] the possibility exists that differences in sexual activity could be accentuated under environmental conditions so as to serve as effective barriers to interbreeding. Furthermore, such differences might be one reason for the failure of *pipiens* to extend its range into warmer areas, and of *fatigans* into colder regions. The purpose of this study was to determine whether high or low temperatures would have a differential effect on mating activities of *C. p. pipiens* and *C. p. fatigans*.

MATERIALS AND METHODS

The following populations of mosquitoes were used: *C. p. pipiens* (Baltimore strain) maintained as a colony for several years; *C. p. pipiens* (Doverbrook strain), established as a colony from larvae collected in the country north of Baltimore in the fall of 1958; *C. p. molestus*, established as a laboratory colony several years ago from eggs laid by a wild caught female in Baltimore; *C. p. fatigans* (Philippine strain); *C. p. fatigans* (Galveston strain).

All temperature-mating experiments were carried out in screen wire cages 1 x 1 x 1 foot in size. The cages were placed in temperature-controlled cabinets for the length of the breeding period. A 15-watt light bulb was placed in each cabinet, and shaded so as to furnish subdued, continuous illumination. Cabinet temperatures were observed by means of mercury, maximum-minimum, and recording thermometers. Variations of about 1°C above or below the stated temperatures occurred; those experiments in which mechanical failure of the control mechanism caused greater fluctuations were discarded.

In setting up an experiment, pupae from the colonies were sexed by size, and 1 to 5 individuals were placed in test tubes for emergence. After emergence, the males and females were transferred to separate holding cages for 2 to 4 days; they were then considered to be sufficiently mature for mating experiments. During the holding and experimental periods, the mosquitoes were fed sugar water and a high relative humidity was maintained by means of rolled toweling inserted in a jar of water inside each cage, and by wet towelling on top of the cage on which a wide-mouth jar containing water was placed in an inverted position to give constant seepage.

Mating activity was measured by the insemination rates which were determined at the end of the breeding period by microscopic examination of the spermathecae of the females. In each experiment, two populations were compared. Thus, if an experiment was run at three temperatures, there were six cages in all, with one for each kind of mosquito at each temperature. There were two to several replicates per experiment. The breeding period and sex ratios remained the same for all replicates of a given experiment; these were changed between experiments, as indicated below.

In studying the effect of changes in illumination on swarming, cages from two colonies were placed side by side in a dark room, beneath an electric drop. The light bulb was about 3 feet above the cages, and the intensity was controlled by means of a manually operated rheostat; the rate of change was 5 units on the indicator scale per minute, representing a change of 2 foot candles at the upper and about 0.2 foot candles at the lower end of the scale. Light intensity was measured on the floor of these cages with

* This work was supported in part by a grant from the National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

a Weston foot-candle meter. Each cage was 1.5 x 1.5 x 2.5 feet in size. The cages were placed in the dark room several hours prior to the beginning of the observation; the room lights were kept on or off depending upon whether it was desired to increase or decrease the illumination. The degree of activity in the cage could not be determined by accurate count of flying insects. However, it was possible to determine when the first individuals began to fly; when 1 or 2 dozen mosquitoes began swarming; when a third or a half of the population was active; and finally when intense swarming appeared to involve almost the entire population. These stages could be detected partly by sight with the aid of a pen-light at lower illumination, used momentarily, and in part by sound. These criteria are crude; nevertheless, it was possible to observe differential activity by such measurements.

EXPERIMENTAL RESULTS

Experiment 1. Comparisons of insemination rates were made between Baltimore *pipiens* and Philippine *fatigans*, at 15, 20, 25, 30 and 35°C. There were 40 females and 20 males in each cage; the breeding period was 48 hours. The results are given in Table 1. At 15°C both populations were inhibited, and in one observation made at 35°C, *pipiens* showed greater inhibition than did *fatigans*. However, at all temperatures, even at the lower range, the tropical Philippine *fatigans* was more active than *pipiens*.

TABLE 1

Insemination rates of C. p. pipiens (*Baltimore strain*) *and* C. p. fatigans (*Philippine strain*)

Temp. (°C)	Mosq.	No. repl.	No. dissect.	% inseminated		p*
				Mean	Diff.	
15	P	4	160	2.5	4.4	—
	F	4	159	6.9		
20	P	4	160	25.0	2.5	—
	F	4	160	27.5		
25	P	8	314	25.8	15.1	<.01
	F	8	320	40.9		
30	P	4	155	14.8	16.6	<.01
	F	4	159	31.4		
35	P	1	40	10.0	14.5	.06
	F	1	39	24.5		

* Based on SE diff. $\% = \sqrt{pq\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$.

TABLE 2

Insemination rates of C. p. pipiens (*Doverbrook strain*) *and* C. p. fatigans (*Philippine strain*)

Temp. (°C)	Mosq.	No. repl.	No. dissect.	% inseminated			p
				Range	Mean	Diff.	
15	P	3	140	11.1–23.9	17.9	13.5	<.01
	F	3	137	16.3–56.1	31.4		
23	P	3	116	10.6–54.5	24.1	13.1	.02
	F	3	142	26.5–48.9	37.3		
35	P	3	129	0– 5.4	2.3	14.5	<.01
	F	3	131	5.0–29.2	16.8		

Experiment 2. In this experiment there were 3 combinations of populations: Doverbrook *pipiens* vs. Philippine *fatigans*, Doverbrook *pipiens* vs. *molestus*, and Philippine *fatigans* vs. Galveston *fatigans*. The mosquitoes were subjected to temperatures of 15, 23, and 35°C; the breeding period was extended to 4 days; and 50 males and 50 females were placed in each cage.

The results presented in Table 2 show that, in general, Philippine *fatigans* was the most active at all temperatures, including the lowest, although occasionally there were reversals between replicates at 15 and 23°C. Table 3 shows *molestus* to be far more active than any other population, at the highest as well as the lowest temperatures. There was no difference between the Philippine and Galveston *fatigans* (Table 4). We are unable to explain why, in the series of replicates presented in Table 4, there was far less mating activity at 15°C than in those involving Philippine *fatigans* in combination with Doverbrook *pipiens* (Table 2). However, they are similar to the response observed at 15°C in Experiment 1. Also unexplained is the greater activity of *pipiens* in the *pipiens-molestus* tests (Table 3) at 15 than at 23°C. It is of interest to note that increasing the number of males and the somewhat greater population densities per cage, as well as lengthening the breeding period, did not markedly increase the insemination rates.

Experiment 3. Swarming activities. Since rather marked differences in sexual activity are exhibited by our colonies, observations were made of the effect of changes in light intensities. Each observation consisted of a comparison of two colony cages, each containing several hundred adults. The air-conditioned dark room was maintained at approximately 25°C. Swarming was considered

TABLE 3

Insemination rates of C. p. pipiens *(Doverbrook strain) and* C. p. molestus

Temp. (°C)	Mosq.	No. repl.	No. dissect.	% inseminated			p
				Range	Mean	Diff.	
15	P	2	97	22.0–38.3	33.4	54.1	<.01
	M	2	96	85.1–93.6	87.5		
23	P	2	95	8.3–14.9	11.6	87.3	<.01
	M	2	94	97.7–100	98.9		
35	P	2	79	0– 4.3	2.5	62.1	<.01
	M	2	82	38.5–88.4	64.6		

TABLE 4

Insemination rates of C. p. fatigans, *Philippine and Galveston strains*

Temp. °C	Mosq.	No. repl.	No. dissec.	% inseminated			p
				Range	Mean	Diff.	
15	Phil.	3	143	0– 2.0	0.7	1.5	.27
	Gal.	3	134	0– 4.2	2.2		
23	Phil.	3	145	42.9–74.5	58.6	2.7	>.32
	Gal.	3	143	44.0–79.1	55.9		
35	Phil.	3	132	16.7–34.9	23.5	6.2	.23
	Gal.	3	138	21.7–38.0	29.7		

to be a more or less continuous flight characterized by agitated lateral and vertical movements. The results are presented in Table 5. Observation 1 was repeated three times; in each case the Philippine *fatigans* began swarming as the light decreased to 2.6 foot candles and continued until the room was completely dark; the Baltimore *pipiens* did not become active until almost complete darkness had set in. At this point the *pipiens* swarm continued while that of *fatigans* subsided. Observation 2 was repeated three times; the Galveston *fatigans* and Doverbrook *pipiens* also showed intense swarming at 0.05 foot candles; again the *fatigans* became quiescent in complete darkness while the *pipiens* swarm continued. In the above experiments, the mosquitoes had been conditioned in the light. In Observation 3, they were kept for several hours in complete darkness. In two replicates, the Galveston *fatigans* became active at 0.05 foot candles, and continued to swarm in the bright light, while Doverbrook *pipiens* remained quiet.

The maximum amount of light produced by the

TABLE 5

Swarming activity of Culex *populations with changing light intensity*

Observation	Ft. candles	Activity: numbers of mosquitoes participating			
		fatigans (Phil)	fatigans (Gal)	pipiens (Balt)	pipiens (Dov)
1	16.5 –2.7	1–2		1–2	
decreasing	2.6 –.7	12		1–2	
white	.6 –.06	36		1–2	
light	.05–0	+++*		12	
	0	0		+++	
2	16.5 –2.7		1–2		1–2
decreasing	2.6 –.06		12–24		12–24
white	.05–0		+++		+++
light	0		0		+++
3	0		0		0
increasing	.05		12		0
white	16.5		+++		0
light					
4	.5 –.16	12	0	0	0
decreasing	.15–.06	24	0	0	0
blue light	.05–0	+++	0	12	12
	0	0	0	+++	+++
5	1.0 –.06	0	0	0	0
decreasing	.05–0	0	0	0	12
red light	0	0	0	0	+++

* +++ indicates an intense swarm.

blue bulb in Observation 4 was 0.5 foot candles. The Philippine *fatigans* began swarming at 0.15 foot candles, and ceased in complete darkness; the Galveston *fatigans* did not respond at all; both *pipiens* populations began swarming at 0.05 foot candles and continued intense activity in complete darkness.

Under a red light, only the Doverbrook *pipiens* responded as complete darkness was attained.

In order to determine whether copulation actually took place during the swarm, a cage of virgin Philippine *fatigans* was induced to swarm for 20 minutes; then the females were divided into two groups and placed separately in pint-sized cardboard containers covered at one end with gauze. There were 61 females in one group and 64 in the other; to the latter, 4 males were added. They were fed sugar water, and after 2

days the females were dissected and examined for the presence of sperm. In the group with 4 males, 14 of 64 females, or 21.8%, were positive. In the other group, 5, or 8.2%, were positive. Thus some insemination did take place during the swarming period.

DISCUSSION

At low temperatures, northern *pipiens* did not show higher insemination rates than did tropical or subtropical *fatigans* populations. Both were inhibited at low temperatures. Both subspecies were also inhibited at high temperatures, but at 35°C the Galveston and Philippine *fatigans* were able to maintain relatively high insemination rates, while only an occasional *pipiens* female was inseminated. As *fatigans* usually showed much greater activity at all temperatures, it does not appear that it is specifically adapted for mating at high temperatures. *C. p. molestus*, which occurs only in the *pipiens* range, was even more active than *fatigans* at high as well as at low temperatures. These differences, then, reflect inherent differences in sexual aggressiveness rather than temperature adaptations.

In a preceding paper Tekle[7] has shown that the inability of *C. p. fatigans* to metabolize its stored nutritive reserves is a possible barrier to the northward extension of its range. Farid[8] suggested that high temperatures may act as a barrier to the southward extension of *C. p. pipiens* through inhibition of sexual activity. Although our results do not give strong support to the hypothesis that environmental temperatures affect geographic distribution of the *Culex pipiens* populations, some responses such as we observed could have the effect of limiting the southward extension of *pipiens* into warmer regions. In such cases, where the populations might be in competition, a reduced activity on the part of *pipiens* with continued high activity on the part of *fatigans* should give the latter population a considerable selective advantage over the former. It must be recognized, of course, that swarming, presumably accompanied by some mating at least, takes place at dusk or night at a time when the temperatures may be expected to drop. Whether this drop is sufficient during the warmest part of the summer in the southern United States to permit *pipiens* to maintain normal sexual activity can be determined only by extensive observations in the field.

The second important environmental factor which affected swarming and mating is illumination. The many observations which have been made on swarming of mosquitoes have shown clearly that change in light intensity is a direct stimulus to swarming flight. There is a difference of opinion as to the biological reasons for swarming. The extensive literature cannot be reviewed here, but we should note Downes[9] conclusion that swarming has two functions: (1) to concentrate the population into small areas, and (2) to bring the sexes together so that copulation can be accomplished.

Now we see in our results a possible mechanism for mating barriers which could keep *pipiens* and *fatigans* populations segregated in a given area. The *fatigans* became active sooner than did the *pipiens* populations as the light intensity was reduced. The peak of the *fatigans* swarming occurred just before dark, whereas that of *pipiens* occurred in darkness. If one adds to this the possible differential effect of temperature, the mating barrier may become important in the maintenance of *fatigans* and *pipiens* as isolated populations in those areas where they coexist. On the other hand, since a given population does seem to respond in varying degrees to stimuli which induce mating, it is probable that at times the swarming and mating activities of *pipiens* and *fatigans* would coincide, resulting in the degree of hybridization which has been indicated by the studies on wild-caught material.

SUMMARY

Mating experiments with several strains of *C. p. pipiens* and *C. p. fatigans* at different temperatures showed that *fatigans* was more active at all temperatures, but was less inhibited than *pipiens* at the highest temperature. With decreasing intensity of illumination, *fatigans* began swarming sooner than did *pipiens*, but ceased shortly after complete darkness was attained, whereas *pipiens* continued to swarm in the dark.

It is concluded that *fatigans* does not exhibit a specific temperature adaptation for mating, but that the higher insemination rates at all temperatures were the result of an inherent sexual aggressiveness. However, such differential activity could be a means of limiting the establishment of *pipiens* in warmer areas, where it would have to compete with the more active *fatigans*. Furthermore, these differences in response to tem-

perature and changes in illumination could be a mechanism for keeping the populations segregated in some areas where they coexist.

REFERENCES

1. Sundararaman, S., 1949. Biometrical studies on integration in the genitalia of certain populations of *Culex pipiens* and *Culex quinquefasciatus* in the United States. *Am. J. Hyg.*, **50:** 307–314.
2. Barr, R. A., 1957. The distribution of *Culex p. pipiens* and *C. p. quinquefasciatus* in North America. *Am. J. Trop. Med. & Hyg.*, **6:** 153–165.
3. Bekku, H., 1956. Studies on the *Culex pipiens* group of Japan. I. Comparative studies on the morphology of those obtained from various localities in the Far East. *Nagasaki M. J.*, **31:** 956–966.
4. McMillan, H. L., 1958. Study of a naturally occurring population intermediate between *Culex p. pipiens* and *C. p. quinquefasciatus*. *Am. J. Trop. Med. & Hyg.*, **7:** 505–511.
5. Rozeboom, L. E., and Gilford, B. N., 1954. Sexual isolation between populations of the *Culex pipiens* complex in North America. *J. Parasitol.*, **40:** 237–244.
6. Rozeboom, L. E., 1958. Hybridization of *Culex pipiens fatigans* Wiedemann from the Philippine Islands with American strains of the *Culex pipiens* group (Diptera: Culicidae). *Am. J. Trop. Med. & Hyg.*, **7:** 526–530.
7. Tekle, A., 1960. The physiology of hibernation and its role in the geographical distribution of populations of the *Culex pipiens* complex. *Am. J. Trop. Med. & Hyg.*, **9:** 321–330.
8. Farid, M. A., 1949. Relationships between certain populations of *Culex pipiens* Linnaeus and *Culex quinquefasciatus* Say in the United States. *Am. J. Hyg.*, **49:** 83–100.
9. Downes, J. A., 1958. Assembly and mating in the biting Nematocera. *Proc. 10th Internat. Cong. Ent.*, **2:** 425–434.

OBSERVATION ON ETIOLOGY OF DIARRHEAL DISEASES IN ARIZONA*

MELVIN H. GOODWIN, JR., DON C. MACKEL, ROBERT S. GANELIN, ROBERT E. WEAVER AND FRED J. PAYNE

Communicable Disease Center, Bureau of State Services, Public Health Service, U. S. Department of Health, Education, and Welfare, Phoenix, Arizona

The studies reported here were undertaken to evaluate the present association of enteric pathogens with acute diarrheal diseases. Observations were made in southern Arizona, an area of the United States where the highest rates of mortality attributed to enteric infections are encountered. Notwithstanding substantial reductions in the two previous decades, diarrheal diseases are still a principal cause of infant mortality in the United States. In the period from 1945 to 1957, the annual number of deaths reported dropped from approximately 12,500 to 5,000. The general rise in living standards, with attendant improvement in medical care, nutritional status, and environmental sanitation, is commonly accepted as a factor contributing to the diminution of diarrheal diseases. The precise effect of such changes in patterns of living on diarrheal diseases is difficult to appraise, especially in the absence of definitive information on the identity and mode of spread of the etiologic agents involved. The justification for empirical application of control measures is, however, substantiated by the results achieved, whether directly or indirectly. Examination of the latest figures available on infant mortality revealed that the annual number of deaths has stabilized at slightly less than 5,000. It seems apparent, therefore, that definitive etiologic and epidemiologic information leading to development of more precise control techniques is necessary to maintain the favorable rate of decline in diarrheal infections.

Impressions from recent studies in Arizona suggested that the rate of *Shigella* isolation from persons with acute diarrhea was significantly less than that reported from studies in southwestern United States during earlier periods when mortality rates were more than twice the current rate.[1] Results from preliminary investigations corroborated these subjective impressions and led to the more extensive observations described below.[2]

* Read at the Eighth Annual Meeting of the American Society of Tropical Medicine and Hygiene, Indianapolis, Indiana, 31 October 1959.

METHODS

During investigations conducted from October, 1957 through February, 1959, efforts were made to establish etiology for representative acute clinical cases of diarrhea for which medical attention was sought. Efforts were made to secure material from various social and economic groups. Specimens were obtained from indigent hospitalized and clinic patients at the Maricopa County Hospital, from the Public Health Service Phoenix Indian Hospital, and from the Public Health Service Indian Hospital at Sacaton, Arizona on the Pima reservation. Some material was also obtained from pediatricians in private practice. All patients with a principal complaint of diarrhea were admitted to the study except those with an associated major illness, or those who received antiparasitic or antibacterial therapy, except penicillin, within the week prior to the onset of their illness. Supplies of containers for specimens, culture media, and storage facilities were provided at each cooperating institution. Specimens were obtained in the following manner, after decision by the responsible physician that a patient was eligible for study. Fecal material obtained by the rectal swab technique was inoculated onto both SS and MacConkey's agar plates. The swab was then placed in tetrathionate enrichment broth which was streaked on brilliant green agar after incubation for 24 hours. Examination and complete serologic identification of *Shigella*, *Salmonella*, *Arizona* and *Escherichia coli* were made according to methods described by Edwards and Ewing.[3] Other groups of Enterobactericeae identified were *Citrobacter*, *Proteus*, *Providence*, *Klebsiella*, *Hafnia*, *Alkalescens-Dispar* and intermediate strains. Provisions were also made for detection and identification of *Pseudomonas aeruginosa*. A portion of the first passed stool specimen was placed in PVA preservative for subsequent parasitologic examination. When feasible, blood specimens were collected both during the acute

TABLE 1

Rates of enteropathogenic Escherichia coli, Shigella, *and* Salmonella *among 630 persons with symptoms of enteric infections during October 1957 through February 1959 in Maricopa and Pinal Counties, Arizona*

	Number examined	Number positive†	Per cent positive	Per cent of cases examined with associated pathogens		
				Esch. coli	*Shigella*	*Salmonella*
Hospitalized	377	242	64	37	29	5
Outpatient	101	51	50	27	30	3
Miscellaneous*	152	65	43	20	13	14
All cases	630	358	57	31	26	7

* Includes persons examined after antibiotic therapy, "possible asymptomatic enteric infections," and patients who developed diarrhea after admission to hospital for reasons other than diarrhea.

† Includes 55 individuals with multiple infections.

TABLE 2

Types of Shigella *identified by months from 630 persons with symptoms of diarrhea during October 1957 through February 1959 in Maricopa and Pinal Counties, Arizona*

Type of *Shigella*	Number of isolations																	
	Total	1957			1958												1959	
		O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F
A2	10								1	2				3	1	3		
A3	4						1		1				2					
B1a	3		1	1			1											
B1b	3							1						2				
B2a	40			1	1	1	3	1	2	6	5	1	7	4	3	3	2	
B2b	3		1				1				1							
B3	24			2		2		3	1			1	1	5	3	6		
B4a	18	1	1	2	1		1			1		4	1	4	1			1
B5	7			1		1	1				1	2			1			
B6	16			1			2					3	2	2	3	1	1	1
C2	8											2	1	3	1	1		
C12	1													1				
D1	25		1		2			1	1		1	1	4	3	5	5	1	
Total	162	1	4	8	4	4	10	6	6	9	8	14	18	27	18	19	4	2

illness and during convalescence. Throat swabs and stool specimens were collected and frozen for study of viruses.

RESULTS

Because of variation in severity of illness, data from hospitalized patients and outpatients were analyzed separately. A "miscellaneous" category, which included persons examined after antibiotic therapy, "possible asymptomatic enteric infections," and patients who developed diarrhea after admission to the hospital for reasons other than diarrhea, was also considered separately. Results of analyses are shown in tables 1 through 7. The data in Tables 4 through 6 do not include information from the miscellaneous group. As indicated in Table 1, recognized enteric bacterial pathogens were detected in 358 (57%) of the 630 cases observed. Multiple infections found in 55 instances were not excluded from this tabulation. The rate of recovery varied from 64% for the hospitalized cases, presumably the most

TABLE 3

Types of enteropathogenic Escherichia coli *identified by months from 630 persons with symptoms of diarrhea during October 1957 through February 1959 in Maricopa and Pinal Counties, Arizona*

Type of enteropathogenic *Esch. coli*	Number of isolations																	
	Total	1957			1958												1959	
		O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F
026	5									2				2	1			
055	37		3	5	1	2	3	1	1	4		1	3	3	3	5	1	1
086	24			3	1	2	1	1	1					2	8	3	2	
0111	56		6	2	2	3	2	1	5	8		1	4	5	6	3	5	3
0112	1													1				
0119	21		2	4	1	2	2	1							1	6	2	
0124	0																	
0125	15		1	2		3				1			2	1	2	3		
0126	31		1	1	2	4			4	2		2	1	5	3	2	3	1
0127	10			2	1	2	1				1			3				
0128	3			1					1					1				
Total	203	0	13	20	8	18	9	4	12	17	1	4	10	23	24	22	13	5

TABLE 4

Distribution of pathogens associated with acute diarrheal disease by race of 478 patients examined from October 1957 through February 1959 in Maricopa and Pinal Counties, Arizona

Race	No. of cases	Per cent of total	Number detected and per cent of total pathogens									
			Esch. coli		*Shigella*		*Salmonella*		Multiple infect.		Unknown	
			No.	%	No.	%	No.	%	No.	%	No.	%
Mexican	128	27	46	37	25	23	2	12	14	31	41	22
Indian	224	47	42	34	60	56	8	50	21	47	93	50
Anglo	62	13	15	12	14	13	3	19	5	11	25	13
Negro	39	8	13	11	6	6	3	19	5	11	12	6
Unknown	25	5	7	6	3	3	0	0	0	0	15	8
All races	478		123	26	108	23	16	3	45	9	186	39

severely ill, to 43% for the miscellaneous group. Enteropathogenic *Esch. coli* was associated with the greatest number of cases, 31%; *Shigella* was isolated from 26%; and *Salmonella* from 7%. Tables 2 and 3 show, respectively, by months the distribution of *Shigella* and types of enteropathogenic *Esch. coli*. *Shigella* types B2a, B3, D1, B4a, and B6 and *Esch. coli* types 055, 0111, and 0126 were predominant, but characteristic seasonal distribution of succession of types was not apparent from the data collected. As shown in Tables 5 and 7, the distribution of cases of unknown etiology was compatible with respect to season and age with that of *Esch. coli* and *Shigella*. Table 7 shows by month of onset, for 583 persons for whom a date was established, the number of patients examined, the number and per cent positive for recognized bacterial pathogens, and the number and rate of *Shigella*, *Salmonella* and enteropathogenic *Esch. coli* associated with acute cases. Analyses by genera of bacterial pathogens also failed to suggest a definite pattern of seasonal occurrence. *Shigella* was, however, relatively more prominent from July through October than during other months, and *Esch.*

TABLE 5

Distribution of etiologic agents associated with diarrheal disease by age of 478 persons examined from October 1957 through February 1959 in Maricopa and Pinal Counties, Arizona

Age	No. of cases	Number and per cent of total pathogens isolated in age groups											
		Per cent of total cases	*Esch. coli*		*Shigella*		*Salmonella*		Multiple infect.		Unknown	
			No.	%	No.	%	No.	%	No.	%	No.	%
1 Month	21	4	7	6	0	0	1	6	1	2	12	6
1–2 Mos	82	17	37	30	9	8	3	19	5	11	28	15
3–6 Mos	137	29	51	41	15	14	5	31	14	31	52	28
7–12 Mos	82	17	14	11	18	17	1	6	11	24	38	20
13–18 Mos	42	9	3	2	20	19	1	6	3	7	15	8
19–24 Mos	11	2	3	2	6	6	0	0	1	2	1	1
2–12 Years	65	14	1	1	30	28	4	25	7	16	23	12
>12 Years	14	3	1	1	5	5	1	6	2	4	5	3
Unknown	24	5	6	5	5	5	0	0	1	2	12	6
All ages	478		123	26	108	23	16	3	45	9	186	39

TABLE 6

Distribution by age of acute cases of diarrhea and of per cent positive for Shigella *in two studies in the Southwest*

Age (years)	Number of cases	Per cent of total cases	Number positive	Per cent positive	Per cent of positive cases
New Mexico 1936–1938*					
<1	86	35	57	66	30
1–2	55	22	43	78	23
2–14	39	16	37	95	20
>14	67	27	49	73	26
Unknown	2	1	2	100	1
All ages	249		188	76	
Arizona 1957–1959					
<1	322	67	42	13	39
1–2	53	11	26	49	24
2–12	65	14	30	46	27
>12	14	3	5	36	5
Unknown	24	5	5	21	5
All ages	478		108	23	

* Data from Hardy and Watt.[1]

coli was relatively more prominent during the fall and winter months.

The distribution of cases and the rate of recovery of bacterial pathogens by race are displayed in Table 4. No pronounced association of an etiologic agent with a specific ethnic group is apparent from these data. *Shigella* were recovered at a slightly higher rate from Indians than would be anticipated if the distribution of these pathogens was completely normal.

The data in table 5 indicate by age groups the distribution of cases studied, the number of pathogens of each genus detected, and the per cent of the total number of pathogens of each genus. Approximately 67% of the patients with acute diarrhea were less than 1 year of age. The rate of enteropathogenic *Esch. coli* in this group was about 34%, which accounted for about 89% of the total number of these pathogens isolated. *Shigella* were isolated from about 13% of persons under 1 year of age; about 39% of those detected occurred in this age group. Too few *Salmonella* were isolated to provide reliable data on distribution. The cases from which no pathogens were isolated did not exhibit a characteristic distribution with respect to age but were generally comparable to the age distribution of the persons examined.

As would be expected for persons of the ages studied, parasites were encountered too infrequently to be considered of etiologic significance. Only one case of diarrhea was attributed to *Entamoeba histolytica*. Other parasites detected by examination of 284 persons revealed 4 infections of *Giardia lamblia*, 3 of *Trichomonas hominis*, and 1 of *Endolimax nana*.

TABLE 7

Number and incidence of enteropathogenic Escherichia coli, Shigella *and* Salmonella *among 583 persons by date of onset during the period November 1957 through February 1959 in Maricopa and Pinal Counties, Arizona*

	No. of persons examined.	No. and per cent positive		Number and incidence of pathogens*					
				Esch. coli		*Shigella*		*Salmonella*	
		No.	%	No.	%	No.	%	No.	%
1957									
November	27	18	67	17	63	5	19	2	7
December	30	17	57	14	47	6	20	2	7
1958									
January	14	8	57	5	36	3	21	1	7
February	26	18	69	17	65	4	15	0	0
March	29	13	45	6	21	8	28	0	0
April	21	9	43	4	19	6	29	0	0
May	36	17	47	13	36	6	17	1	3
June	35	21	60	15	43	8	23	3	9
July	22	8	36	0	0	8	36	0	0
August	34	24	71	6	18	14	41	7	21
September	46	26	57	9	20	18	39	4	9
October	71	42	59	22	31	26	37	3	4
November	87	40	46	23	26	17	20	5	6
December	68	42	62	20	29	21	31	7	10
1959									
January	18	14	78	10	56	3	17	3	17
February	19	9	47	8	42	2	11	1	5
	583	326	56	189	32	155	27	39	7

* Includes multiple infections.

Clinical and epidemiological aspects of this study, report of virologic work, and analyses of associated bacterial agents for which etiologic relations were not apparent will be presented elsewhere.

DISCUSSION

Comparison of prevalence of enteric pathogens conducted by different investigations, at different times, and in different areas is at best a tenuous procedure. It is apparent, however, that during the present study *Shigella* was associated less prominently with cases of acute diarrheal diseases than was observed by Hardy and Watt.[1] As the areas represent different ecologic types, the differences observed may have resulted from environmental factors which favor higher rates of *Shigella* in one area than in another. Comparison of antecedent mortality statistics indicates, however, that the two areas have had similar rates during the period for which simultaneous records are available. Comparable decreases in mortality have occurred in both places. It appears likely, therefore, that the differences observed may reflect actual changes in the relative prevalence of *Shigella* associated with diarrhea. A comparison of the two studies was made on the possibility that it would indicate evolutionary changes in the significance of etiologic agents associated with diarrhea rather than inherent differences in diarrheal diseases in the two areas. The data available do not permit broad generalization concerning other areas, however.

Some comparative data from the two studies are summarized in Table 6. The data considered from the Hardy and Watt series are from patients designated as severely ill. Information from the Arizona study was obtained from all patients who sought treatment for acute diarrhea; the miscellaneous group of Table 1 was not included. The

methods for obtaining cases for study differed in some respects in the two studies, but it is unlikely that these differences would have resulted in selection of incomparable samples. Comparison of data from these studies indicates significant differences in age distribution and etiology of acute diarrheal diseases. In the Arizona series, *Shigella* was not associated with as large a proportion of illness as observed in the New Mexico study. The over-all infection rate of *Shigella* in the study of Hardy and Watt was about 75%. Highest rates (95%) were observed in the 2 to 14 year age group and the lowest (66%) in the infants under 1 year of age. In contrast, the over-all infection rate for *Shigella* was 23% in the present study.

In the Arizona study, infants under 1 year of age accounted for 67% of the acutely ill patients for whom treatment was sought. In the series observed by Hardy and Watt, infants were prominent, but children under 1 year of age accounted for only 35% of the cases observed. In the New Mexico study, persons more than 14 years of age comprised 27% of the cases studied; but, in the Arizona series, persons more than 12 years of age contributed only 3% of the cases. Information from other studies in communities of southern Arizona corroborates the relative prevalence of diarrhea by age groups observed during this etiology study.

These observations confirm the general impression that the relative prominence of *Shigella* has diminished and that severe diarrhea is rare in persons of school age and older. The present patterns of occurrence may be characteristic of situations where significant control of bacillary dysentery has been achieved.

Since extensive bacteriologic and parasitologic examinations failed to demonstrate etiological agents in 43% of the cases examined, the possibility of an occult etiologic agent should be considered. Examination of serial specimens probably would have increased the rate of recovery of bacterial pathogens. Hardy and Watt were able to increase the rate of *Shigella* isolation by 45% when four specimens rather than one were examined. This does not alter the validity of the above comparison, however. In Hardy and Watt's series, 38% of the severely ill patients had only one examination. Even if isolations in the present study were increased by 45%, which is unlikely considering the superiority of techniques now available, the difference between results of the two studies would still be significant.

The cases from which etiologic agents were not recovered did not exhibit a characteristic seasonal or age distribution. As shown in Table 5, the distribution of cases from which isolations were not made was almost identical with the per cent of total cases in the various age groups. Comparison of the monthly distribution of enteropathogenic *Esch. coli* and *Shigella*, and of cases without associated pathogens, failed to suggest that the cases of undetermined etiology did not concur with the cases from which pathogens were isolated. Presumption of unrecognized, occult etiologic agents is not necessary to explain the distribution of the cases from which pathogens were not isolated. The evidence available does not, however, preclude the possibility that undetected pathogens are involved.

The prominent occurrence of enteropathogenic types of *Esch. coli* raises questions concerning the etiologic significance of these pathogens in sporadic cases of diarrhea. The causal relationship between recognized enteropathogenic *Esch. coli* and diarrhea usually has been established on the basis of epidemiologic evidence obtained during out-breaks of infantile diarrhea. Most workers accept evidence of pathogenicity of *Esch. coli* in such instances, but the evidence of causal relation of the organism in sporadic cases is not conclusive. It is recognized that the concurrence of symptoms and an associated organism is not itself evidence of pathogenicity. Some evidence is available, however, to support the etiologic role of certain *Esch. coli*. The pathogenicity of four types (055:B5, 086:B7, 0111:B4, 0127:B8) was corroborated by experimental infection of humans.[4, 5] Furthermore, Thomson[6] studied the distribution in the gut of some enteropathogenic *Esch. coli* in cases of illness attributable to these infections. Colonization of the entire gut by specific enteropathogenic types, similar to the syndrome of cholera, and concentration of organisms in the gall bladder were reported.

More extensive epidemiologic data are needed to show conclusively that these bacteria occur more frequently in persons with symptoms of diarrhea than in persons who do not have diarrhea. However, in a limited study, which will be reported elsewhere in detail, enteropathogenic *Esch. coli* was recovered at a signifi-

cantly lower rate from siblings and other household contacts without symptoms of diarrhea than from the member of the household with acute diarrhea. This observation is supported by results from other studies; *e.g.*, Thomson *et al.*,[7] Hallman *et al.*,[8] and Cooper *et al.*[9] Because of the pronounced age specificity of these pathogens, however, definitive data have not yet been obtained from study of household contacts in the present study.

About 67% of patients observed in this study were infants less than 1 year old. Persons of this age are more likely to develop serious illness than are older persons. Their prognosis may be less favorable also because of the inclination toward rapid dehydration. These conditions obviously contribute to development of severe illness and to the high mortality attributed to infantile diarrhea. The question may be raised as to whether or not the associated pathogens are responsible for precipitating the symptoms observed in such cases. The pathogens may have been benign associates or may have become established when conditions favorable for their invasion of the gastrointestinal tract resulted from other conditions. Evidence is not sufficient to resolve these points. But it is essential that the nature of the association between symptoms and pathogens be elaborated for intelligent application of control measures.

SUMMARY

A total of 630 persons with symptoms of acute diarrhea was studied to evaluate the association between recognized enteric pathogens and diarrhea. Approximately 67% of persons for whom treatment was sought were less than 1 year of age. Recognized bacterial pathogens were isolated by a single examination from 57% of the cases. *Shigella* were recovered from 26%, enteropathogenic *Esch. coli* from 31%, and *Salmonella* from 7%. About 90% of all enteropathogenic *Esch. coli* recovered were from infants less than 1 year old; about 40% of the *Shigella* isolations were from this group. The cases from which etiologic agents were not recovered showed no characteristic distribution with respect to age of patients or month of occurrence.

Results differ from earlier studies in the southwest in that the rate of association of *Shigella* with acute diarrhea was lower and relatively few persons other than infants were found with acute enteric symptoms.

ACKNOWLEDGMENT

Grateful acknowledgment is made of the contributions of Doctor Thomas H. Bate, Medical Director, and Doctor Luis Tan, Senior Resident in Pediatrics, Maricopa County Hospital; of Doctor William S. Baum, Area Medical Officer, and Doctor Leland J. Hanchett, Medical Officer in Charge, Phoenix Indian Hospital, Division of Indian Health, Public Health Service; and of their numerous associates who supplied material and aided this study in many ways.

REFERENCES

1. Hardy, A. V., and Watt, J., 1945. Studies of the acute diarrheal diseases. XII. Etiology. *Pub. Health Rep.*, **60**: 57–66.
2. Coleman, P. J., Richards, C. S., DeCapito, T. M., and Maier, P. P., 1959. Observations on the etiology of diarrhea in two Arizona communities. To be published.
3. Edwards, P. R., and Ewing, W. H., 1955. *Identification of Enterobacteriaceae.* Burgess Publishing Co., Minneapolis.
4. Ferguson, W. W., 1956. Experimental diarrheal disease of human volunteers due to *Escherichia coli*. *Ann. New York Acad. Sc.*, **66**: 71–77.
5. Braun, O. H., and Resemann, G., 1952. Über das Vorkommen von *E. coli* 026:B6 und 086 bei der Säuglings Enteritis. *Klin. Wchnschr.*, **30**: 853–854.
6. Thomson, S., 1955. The role of certain varieties of *Bacterium coli* in gastroenteritis of babies. *J. Hyg.*, **53**: 357–367.
7. Thomson, S., Watkins, A. G., and Gray, O. P., 1956. *Escherichia coli* gastroenteritis. *Arch. Dis. Childhood*, **31**: 340–345.
8. Hallman, N., Rantasalo, I., Tuuteri, L., and Kotilainen, M., 1954. Incidence of "dyspepsia coli" (026; 055; 0111) in sporadic infantile gastroenteritis. *Ann. paediat. Fenniae*, **1**: 27–33.
9. Cooper, M. T., Keller, H. M., and Walters, E. W., 1957. Comparative frequency of detection of enteropathogenic *Escherichia coli*, *Shigella*, and *Salmonella* in rectal swab cultures from infants and young children. *Pediatrics*, **19**: 411–423.

STUDIES ON PARALYTIC POLIOMYELITIS IN CAIRO, U.A.R.,* 1957–1958

THOMAS G. AKERS, NEMAT HASHEM†, AND M. SABET MAHDY

United States Naval Medical Research Unit No. 3, Cairo, U.A.R.

Serological surveys in French Morocco,[1] Israel[2] and Saudi Arabia[3] indicated that poliomyelitis was endemic and occurred primarily in the infant population. In Cairo, U.A.R., similar trends were observed by Paul *et al.*[4] and Goldblum and Melnick.[5] Poliovirus isolations by Ward,[6] Melnick,[7] and Horstmann[8] showed that the virus was recoverable in the Cairo area. In recent years there has been an increase in reported paralytic cases, evidence of either growing awareness or worsening of the problem. This availability of a large number of paralytic cases afforded opportunity for further study of endemic poliomyelitis in the area.

Herein are reported the results of a poliovirus isolation survey of 830 paralytic patients reporting to the Children's Hospital, Kasr El Ainy Faculty of Medicine, Cairo. Poliovirus types responsible for paralytic poliomyelitis and their relationship to age, sex, and seasonal distribution within the survey group were determined. As far as it is known, this is the first systematic study to be made of poliovirus distribution throughout an entire season (July 1957 through June 1958) in the Middle East.

MATERIALS

Paul *et al.*[4] derived their incidence data for poliomyelitis in Cairo by reviewing case histories at the Kasr El Ainy Children's Hospital. This hospital is still the most active poliomylitis center in Cairo and was again utilized for this study. Approximately 75% of poliomyelitis patients seen at this particular hospital were from Urban Cairo; the remaining were from Lower or Upper Egypt, or the Canal Zone. The majority were medical indigents. Patients reporting for the first time were directed to one of us (N.H.). Those clinically diagnosed as having paralytic poliomyelitis with onset within 15 days were selected for this study. Clinical criterion for selection was based on the presence of flaccid paralysis. "Onset interval" denoted the time from beginning paralysis to reporting at the hospital. An arbitrary time limit of 15 days was necessary as the exact onset date was ambiguous in many cases. Diminished virus excretion after the second week of paralysis was expected to render a single rectal swab virus recovery effort too unrewarding.

Paul *et al.*[4] had observed that, "paralytic patients usually appeared long after the acute onset, that is, from one week to one year." This observation still holds true. Of 1741 cases of paralytic poliomyelitis reporting to the Children's Hospital during the study year, 622 or 37.7% had an onset interval of 30 days or more. Further analysis showed that 911 or 52.3% of the total paralytic patients had onset intervals of 15 days or more. Thus, of the 1741 patients, only 830 were selected for virus isolation attempts. For this group the mean onset time prior to reporting to the clinic was 7 to 8 days.

All clinical patterns of paralytic poliomyelitis were encountered in the 830 selected patients. However, certain clinical syndromes prevailed over others. Isolated spinal paralysis was exhibited by 85.9% of the selected patients. Bulbospinal paralysis, polioencephalobulbomyelitis or polioencephalomyelitis, and polioencephalitis accounted for 7.6, 5.4, and 1.1% of the studied cases. Patients yielding no virus or viruses other than polio exhibited identical clinical syndromes. No correlation was observed between clinical patterns, age, and associated poliovirus types. Rickets, iron deficiency anemia, and hypoproteinemia, although observed frequently in the study group, had no detectable effect on the patterns of clinical poliomyelitis. No variance in clinical syndromes existed in

* From Research Project NM 52 05 03, Bureau of Medicine and Surgery, Navy Department, Washington, D. C. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

† Guest investigator, NAMRU #3, Lecturer in Pediatrics, Kasr El Ainy Children's Hospital, Cairo University, Cairo, U.A.R.

socioeconomic levels; 22 children from the highest socioeconomic strata showed patterns of occurrence with respect to age, and grade and extension of paralysis identical with those of the indigents.

METHODS

Rectal swabs. Single rectal swab specimens were collected from the selected paralytic patients. Immediately upon collection, swabs were placed in screw-capped vials containing Earle's balanced salt solution (BSS) with antibiotics (500 units penicillin per ml, 200 μg streptomycin per ml, and 100 units of nystatin per ml) supplemented with lactalbumin hydrolysate at a final concentration of 0.1%. After refrigerated transport to the laboratory, swab specimens were stored at -20°C for a maximum period of 6 months until processing and testing. Swabs were prepared for testing according to the method suggested by Paul and Melnick.[9] Kelly hemostats were substituted for syringes for expressing the fluids from the swabs. Collected fluids were centrifuged in a Servall (Type SS-1) centrifuge at 10,000 rpm (R.C.F. = 11,800 $\times$ G) for 20 minutes.

Poliovirus. Poliovirus type 1 (Brunhilde) and type 2 (Lansing) were obtained from the American Type Culture Collection. Poliovirus type 3 (Saukett) was kindly sent by Dr. Dorothy Horstmann, of Yale University School of Medicine. These prototype strains were employed in the production and titration of immune sera required for screening and final typing of viruses isolated.

Production of immune sera. Polio immune sera were produced according to the method of Horstmann[10] by immunizing polio antibody-free *Cercopithecus aethipa sabaeus* (African green monkey) with 3 weekly intramuscular injections of 1.0 ml aliquots of a formalinized virus suspension (poliovirus infected tissue culture fluid with a titer of at least 10^6 TCD_{50} per ml inactivated with formalin at a final concentration of 0.25%; holding at 4°C for 96 hours). After the 4th week, 3 weekly 5.0 ml intramuscular inoculations of a virus-adjuvant mixture,[11] were administered, and subsequent bleeding was commenced during the 8th week. Immune sera so produced and diluted 1 to 25 were type-specific and consistently neutralized at least 1,000 tissue culture infective doses ($TCID_{50}$).

Tissue culture. Using Rappaport's[12] technique, kidney (MK) cultures were produced from locally obtained African green monkeys. Growth medium consisted of Tris-buffered 0.5% lactalbumin hydrolysate in Earle's BSS with 2.5% lamb serum and with antibiotics as follows: 200 units penicillin per ml, 100 μg streptomycin per ml, and 50 units of nystatin per ml. Maintenance medium was the same except that the pH was changed from 7.2 to 7.8 by altering the amount of hydrochloric acid used during media preparation. Four confluent MK cultures were inoculated with 0.2 ml aliquots of each swab supernate; cultures were observed daily for 8 days and those showing cytopathogenic effects (CPE) were subcultured in MK cultures prior to inoculation of HeLa cells[13]. MK cultures without CPE were not further subcultured.

HeLa cells (Gey) were kindly sent by Dr. Mary McClain, University of California. Cells were grown in 8 oz prescription bottles or Carrel flasks (T-60) using a growth medium which was a modified combination of Eagle's[14] and Lepine's[15] media. Earle's BSS at a pH of 7.8 containing 0.25% Difco trypsin was used when harvesting cells. By hemocytometer count, cell suspensions were adjusted to approximately 50,000 cells per ml prior to dispensing into tubes. After 48 to 72 hours at 37°C the cells were usually confluent and were washed twice with Earle's BSS supplemented with 0.1% lactalbumin hydrolysate. Thereafter the cultures were maintained with M-199 (Microbiological Associates, Bethesda, Maryland) supplemented with 0.3% dextrose and 3.0% inactivated normal horse serum. HeLa cells so cultivated were utilized in preliminary neutralization tests.

Neutralization tests. Type specific polio immune serum, diluted 1 to 25 was mixed with an equal amount of infective tissue culture fluid diluted 1 to 100. After incubation of the virus-serum mixture at 37°C for 1 hour, 0.15 ml of the mixtures were inoculated into a duplicate set of confluent HeLa cell cultures. If type specific neutralization failed to occur within 5 days the test was repeated using 100 $TCID_{50}$ of the virus suspension mixed with each of the 3 types of immune serum as well as with a pool of these 3 sera. After incubation, 0.1 ml aliquots of the mixtures were inoculated into duplicate sets of confluent MK cultures. Agents which elicited a cytopathic effect within 7 days, despite mixture

with the pooled antisera, were considered as viruses other than polioviruses.

RESULTS

Poliovirus isolations. Viruses were isolated from 487 or 59.9% of the 830 rectal swabs. Of these, 447 were identified as polioviruses. The remaining 50 agents were not neutralized by polio immune sera and were considered to be enteroviruses other than polio. The poliovirus type distribution is presented in Table 1. Poliovirus type 1 was the most prevalent type during the survey year.

Age distribution. Table 2 indicates the age distribution for the 830 paralytic poliomyelitis patients selected for virus isolation attempts. Approximately 97% of the selected patients were under 30 months of age. The age distribution was similar for patients yielding and not yielding enteroviruses. Table 3 represents the tabulation of the 447 paralytic patients from whom a poliovirus was isolated. The majority (419 or 93.7%) were in the age range from 7 to 30 months. The largest number of cases (37.6%) occurred within the 7 to 12 month group, followed by the 13 to 18 month group (30.2%), and finally by the 19 to 24 month old infants (19.2%). In all, 41% of the patients were under 1 year of age.

This infantile nature of poliomyelitis in Cairo usually meant that the afflicted individual was the youngest child in the family. Of 273 paralytic patients on whom complete family histories were available, 91.3% involved the youngest child, and the remaining 8.3% were the next to youngest.

Sex distribution. Of the 447 poliovirus-yielding patients, 266 or 59.5% were male infants. This predominance in male infants was not affected by age, poliovirus type, or season.

Seasonal distribution. Figure 1 illustrates the seasonal distribution for the 447 laboratory confirmed patients. There is little fluctuation in

TABLE 1

Incidence of three types of poliovirus among cases of paralytic poliomyelitis observed in Cairo, U.A.R., in 1955 and in 1957–1958

Poliovirus types	1955 cases*		1957–1958 cases	
	Number	%	Number	%
Type 1.....	49	38.0	374	61.3
Type 2.....	56	43.4	139	31.1
Type 3.....	24	18.6	34	7.6
Total.....	129	100.0	447	100.0

* From Horstmann[3]

TABLE 2

Age distribution of 830 paralytic poliomyelitis patients observed in Cairo, U.A.R. during 1957–1958 with onset less than 15 days prior to rectal swab sampling for enteroviruses according to results of virus isolation attempts

Age in months	Total paralytic patients			Enteroviruses isolated								
				Polioviruses			Other			None		
	No.	%	Cum. %	No.	%	Cum. %	No.	%	Cum. %	No.	%	Cum. %
0–6	37	4.5	4.5	16	3.6	3.6	4	8.0	8.0	17	5.1	5.1
7–12	301	36.3	40.7	168	37.6	41.2	19	38.0	46.0	114	34.2	34.2
13–18	248	29.9	70.6	135	30.2	71.4	13	26.0	72.0	100	30.0	69.4
19–24	147	17.7	88.3	86	19.2	90.6	9	18.0	90.0	52	15.6	85.0
25–30	50	6.0	94.3	30	6.7	97.3	1	2.0	92.0	19	5.7	90.7
31–36	24	2.9	97.2	6	1.3	98.7	3	6.0	98.0	15	4.6	95.2
37–42	8	1.0	98.2	2	.5	99.1				6	1.8	97.0
43–48	8	1.0	99.2	4	.9	100.0				4	1.2	98.2
49–54	1	.1	99.3							1	.3	98.5
55–60	5	.5	99.9				1	2.0	100.0	4	1.2	99.7
61–66	1	.1	100.0							1	.3	100.0
Total.....	830			447			50			333		

TABLE 3

Age distribution of 447 paralytic poliomyelitis patients from whom a poliovirus was isolated, Cairo, U.A.R., 1957–1958, by type of virus

Age in months	Patients yielding poliovirus type											
	Total			I			II			III		
	No.	%	Cum. %	No.	%	Cum. %	No.	%	Cum. %	No.	%	Cum. %
0–6	16	3.6	3.6	10	3.7	3.7	2	1.4	1.4	4	11.8	11.8
7–12	168	37.6	41.2	99	36.1	39.8	58	41.7	43.2	11	32.4	44.1
13–18	135	30.2	71.4	84	30.7	70.4	42	30.2	73.4	9	26.4	70.6
19–24	86	19.2	90.6	50	18.2	88.7	30	21.7	95.0	6	17.6	88.2
25–30	30	6.7	97.3	20	7.3	96.0	6	4.3	99.3	4	11.8	100.0
31–36	6	1.3	98.7	5	1.8	97.8	1	.7	100.0			
37–42	2	.5	99.1	2	.7	98.5						
43–48	4	.9	100.0	4	1.5	100.0						
Total.....	447			274			139			34		

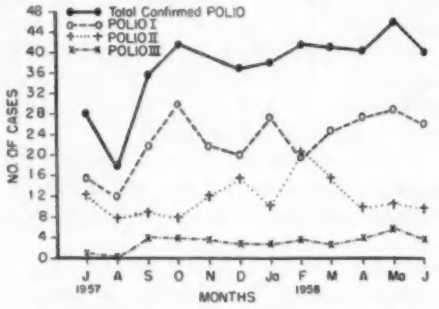


FIG. 1. Seasonal distribution for 447 confirmed cases of paralytic poliomyelitis, Cairo, U.A.R., 1957–1958.

the number of positive cases with the exception of the month of August. The apparent decrease during this month was most probably due to the absence of the collaborating physician on vacation, with some decrease in emphasis and collection of materials.

Location of cases in Cairo. Metropolitan Cairo, including the two contiguous western census districts of Embaba and Giza, has an urban population of approximately 3.5 million according to the 1957 census. Of the 447 confirmed poliomyelitis patients, 340 were residents of metropolitan Cairo; the remaining 107 were from Upper or Lower Egypt, the Canal Zone (including Suez), or their residence was undetermined. For the sake of brevity, the 23 census zones which comprise the areas within urban Cairo, have been consolidated into five sectors, North, East, South, West, and Central Cairo. Table 4 indicates the sector of residence for the 581 selected cases with residences in urban Cairo as well as the population census for each area. When computing rates per 100,000 population one derives a rate of 9.8 for laboratory confirmed cases and 16.8 for the selected cases. These rates would be higher if account is taken of the more than 50% of the reporting patients who were not studied due to onset intervals longer than 15 days. Thus the above calculated rates are minimal.

Although age distribution figures were not available for the 1957 census, it was estimated by the Census Bureau that 13.9% of the population was in the 0 to 4 year old range, giving a

TABLE 4

Paralytic poliomyelitis cases by location within Cairo, U.A.R., 1957–1958

Area	Population	Positive cases	Negative cases	Pos. cases/ 100,000	Total selected cases/ 100,000
North Cairo..	731,203	86	62	11.8	20.2
East Cairo....	903,740	63	46	7.0	12.1
South Cairo...	299,676	34	23	11.3	19.0
West Cairo....	822,960	63	53	7.7	14.1
Central Cairo.	702,630	94	57	13.4	21.5
Total.....	3,460,209	340	241	9.8	16.8

0 to 4 year old population of approximately 480,000 in metropolitan Cairo. From the 340 confirmed paralytic cases a rate of 70.7 per 100,000 is derived and for the 541 selected patients a rate of 112.6.

Within urban Cairo some sectors had more paralytic poliomyelitis than others. This can be seen in Table 4. Within these sectors specific districts could be singled out by their abundance of polio cases. The specific districts in question were Sayeda Zeinab, Mouski, Shoubra, and Giza, located in Central (Sayeda Zeinab and Mouski), North, and West Cairo respectively. Of the 340 confirmed cases from Cairo proper, 44% were from the forementioned districts which contained only 21.2% of the total population. The rate per 100,000 population for paralytic poliomyelitis for the selected cases was 36.3 and for the confirmed 20.5. For the 0 to 4 year old population within these districts the selected rate would be 245.5, and confirmed at 147.3. The seasonal distribution of cases in these districts is quite consistent with the seasonal trend observed for the total cases studied.

Seventy eight confirmed cases were from areas outside of Cairo proper, and were accordingly placed into three categories, that is Lower or Upper Egypt and the Canal Zone (including Suez). No rates were derived but the age and seasonal distribution was similar to that of urban Cairo.

DISCUSSION

The observation that poliovirus type 1 was the most prevalent during the study year indicates a marked change from the survey made in Cairo during 1955–1956 by Horstmann *et al.*[16] and Hashem.[17] The poliovirus type distribution for 129 paralytic cases studied during that period is presented in Table 1. Thus, during the interval of time between these two studies, there occurred a shift from poliovirus type 2 to poliovirus type 1 predominance. This would seem to indicate a shift of predominant types with the passage of time; i.e., the poliovirus type distribution in an endemic area is not always static. The present distribution is similar to that reported by Kono *et al.*[18] in Japan and in general compares with that found in most temperate areas.

The age distribution emphasized the truly infantile nature of the disease in Cairo. The distribution of 41% of the cases under 1 year of age is again very similar to the 46% finding in Tokyo.[18] Figure 2 compares the distribution of selected cases to the distribution of cases reported in the Southern United States by Hall *et al.*[19] The variance perhaps reflects the difference in socioeconomic and related hygienic levels. Obviously, any mass poliomyelitis immunization program in Cairo should emphasize the need for vaccination at the earliest possible age. In this regard it should be pointed out that all available data are derived from children in the poorest socioeconomic level and that children in higher socioeconomic levels may escape the disease until older.

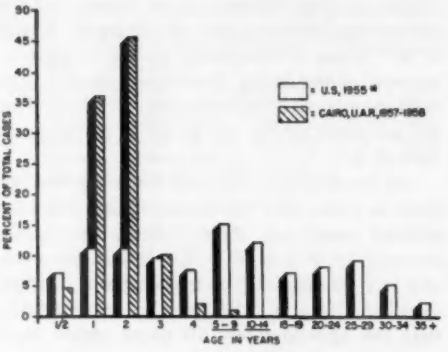


FIG. 2. Percentage distribution of paralytic poliomyelitis by age for Southern United States (reported by Hall *et al.*[19]) and Cairo, U.A.R.

Analysis of the cases by sex revealed that 59.5% were males, which is similar to the 61.3% reported for Cairo by Abdel Khalik *et al.*[20] This male predominance is also similar to that reported by Hall *et al.*[19] in the United States during 1955.

The factors which may predispose the male infant to poliomyelitis are complex and more complete census figures relating the age distributions for the Cairo population as a whole are necessary before further elucidation is possible. However, in the Middle East one might attribute the native mores of favoring male infants as contributing to this predominance. In this study it was observed that age distributions as well as onset intervals were almost identical for both males and females indicating that no particular preference was being placed on either sex.

With regards to seasonal distribution of poliomyelitis in Cairo, the monthly number of paralytic poliomyelitis patients reporting to the

Children's Hospital was uniform throughout the greater part of the survey year. Abdel Khalik *et al.*[21] noted a diminution in the number of reported cases during the winter months (December, January, and February) in Cairo. This did not occur during the period of our study in 1957–1958.

It is interesting to note that the 0 to 4 year old rates in Cairo (70.7 for confirmed and 112.6 for selected cases) are about 4 times the figures reported by Hall *et al.*,[19] that is 24.6 for males and 18.7 for females. The hyperendemic districts in urban Cairo in 1952–1958 had rates (245.5) that are approximately 10 times higher than those reported for the same age group in Southern U. S. in 1955.[19]

SUMMARY

Of the 830 poliomyelitis patients with paralysis of 15 days duration or less reporting to the Children's Hospital, Cairo, U.A.R., from July 1957 through June 1958, 497 yielded enteroviruses, 447 of which proved to be polioviruses. Poliovirus type 1 was the dominant type in 1957–1958 while poliovirus type 2 was dominant in Cairo in 1955. Seasonal distribution for the 447 confirmed cases was uniform throughout the greater part of the year. Ninety-four per cent of the cases occurred in children between 7 and 30 months of age, most of these affecting the youngest child in a family. Although certain districts within Cairo proper accounted for more paralytic cases than others, no explanation was available as to the reason. The incidence of paralytic poliomyelitis was at least 4 times higher in the 0 to 4 year-old population of Cairo in 1957–1958 than in similar age groups in Southern United States in 1955. The results reported herein are in agreement with other observations elsewhere in the Middle East, and all tend to emphasize one point: if poliomyelitis is to be successfully controlled in the Middle East, immunization must be completed at the earliest possible age.

ACKNOWLEDGEMENTS

The authors are grateful to Lowell D. Wilson, Malvin W. Smith, and John P. Hresko for technical assistance. We also wish to acknowledge the interest and encouragement of Captain John R. Seal, MC, USN, Director, U. S. Naval Medical Research Unit No. 3, Cairo, U.A.R. Special acknowledgement is due Dr. Stewart H. Madin, University of California, Berkeley, for the excellent training opportunity afforded T. Akers while previously detailed to U. S. Naval Medical Research Unit No. 1, Berkeley, California.

REFERENCES

1. Paul, J. R., and Horstmann, D. H., 1955. A survey of poliomyelitis antibodies in French Morocco. *Am. J. Trop. Med. & Hyg.*, **4**: 512–524.
2. Bernkopf, H., Medalie, J., and Yekutiel, M., 1957. Antibodies to poliomyelitis virus and socioeconomic factors influencing their frequency in children in Israel. *Am. J. Trop. Med. & Hyg.*, **6**: 697–703.
3. Payzin, S., and Murray, E., 1957. Note on antibodies to poliomyelitis in the sera of residents of Eastern Saudi Arabia. *Am. J. Trop. Med. & Hyg.*, **6**: 704–706.
4. Paul, J. R., Melnick, J. L., Barnet, V. W., and Goldblum, N., 1952. A survey of neutralizing antibodies to poliomyelitis virus in Cairo, Egypt. *Am. J. Hyg.*, **55**: 402–413.
5. Goldblum, N. and Melnick, J. L., 1952. Complement-fixing antibodies to type 2 (Lansing) poliomyelitis virus in a normal population of a subtropical area. *J. Exper. Med.*, **96**: 175–185.
6. Ward, R., 1952. Poliomyelitis and Coxsackie viruses from Egyptian flies. *Fed. Proc.*, **11**: 486.
7. Melnick, J. L., and Agren, K., 1952. Poliomyelitis and Coxsackie viruses isolated from normal infants in Egypt. *Proc. Soc. Exp. Biol. & Med.*, **81**: 621–624.
8. Horstmann, D. M., 1955. Endemic virus infection in Egypt: Isolation of poliomyelitis virus and other tissue culture pathogenic agents from infants. *Fed. Proc.*, **14**: 466.
9. Paul, J. R., and Melnick, J. L., 1956. *Diagnostic Procedures for Virus and Rickettsial Disease*, T. Francis Jr., ed., New York, American Public Health Association. pp. 53–90.
10. Horstmann, D. M., 1955. Personal communication to M. Sabet.
11. Schmidt, N. J., and Lennette, E. H., 1956. Modification of the homotypic specificity of poliomyelitis complement fixing antigens by heat. *J. Exper. Med.*, **104**: 99–120.
12. Rappaport, C., 1956. Trypsinization of monkey-kidney tissue; an automatic method for the preparation of cell suspensions. *Bull. World Health. Organ.*, **14**: 147–166.
13. Gey, G. O., Coffman, W. D., and Kubick, M. T. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.*, **12**: 264–265.
14. Eagle, H., 1955. Nutritional needs of mammalian cells in tissue culture. *Science*, **122**: 501–504.
15. Lépine, P., Slizewicz, P., Daniel, P., Paccaud, M., 1956. Culture of cells in a vitaminized casein hydrolysate medium. Societé Française de Microbiologie, meeting of March 1, 1956.
16. Horstmann, D. M., Frothingham, T., and Saigh, A., 1955. Studies on poliomyelitis in Egypt (Unpublished data).

17. HASHEM N., 1956. Enteric viruses among Egyptian infants and children. *Gaz. Egypt. Pediat. A.*, **4**: 335–348.

18. KONO, R., ASHIHARA, Y., KAWA KAMI, K., MATSUMIYA, T., HIRAYAMA, M., KUBOTA, H., AND TATSUMI, M., 1958. Studies on poliomyelitis in Japan. I. Isolation of poliovirus and the age distribution of neutralizing antibodies in urban populations. *Am. J. Hyg.*, **67**: 135–153.

19. HALL, W. J., NATHANSON, N., AND LANGMUIR A. D., 1957. The age distribution of poliomyelitis in the United States in 1955. *Am. J. Hyg.*, **66**: 214–234.

20. ABDEL KHALIK, A. K., RAGAB, M. M., AND SAKR, R., 1954. Incidence of poliomyelitis in Egypt. *Gaz. Egypt. Pediat. A.*, **11**: 200–208.

21. ABDEL KHALIK, A. K., ABDIN, Z. H., 1954. Some aspects of poliomyelitis in Egypt. *Gaz. Kasr El Ainy Fac. Med.*, **20**: 1–10.

BOOKS RECEIVED

Salud Publica de Mexico, by José Alvarez Amézquita, M.D., and Editorial Staff. Epoca 5, Vol. 1, No. 1, 240 pages, illustrated. Mexico, D. F., R. M., Secretaria de Salubridad y Asistencia, 1959.

Assistance Technique en Médecine Africaine. by René Devignat, Directeur Écoles d'Assist'ce Méd., e de Méd. Trop. Elisabethville, Congo, Belg. 1st edition, 167 pages + xv pages, illustrated. Paris, Masson et Cie, 1958. 1.600 fr.

Annual Report, City Medical Health Officer, City of Durban, South Africa. 1958, by G. D. English, M.B., Ch.B., D.P.H., O.T.M. & H. 135 pages, illustrated. Durban, Durban City Health Department (Mimeograph), 1959.

Report of the First Institute on Clinical Teaching: 6th AAMC Teaching Institute, by Helen H. Gee and Julius B. Richmond, Planning Committee, and E. S. Nourse, editors. 233 + xxx pages, illustrated. Evanston, Ill., Association of American Medical Colleges, 1959. Cloth $3.00, paper $2.00.

Water Supply for Rural Areas and Small Communities, by Edmund G. Wagner, Rio de Janeiro, and J. N. Lanoix, Geneva. 1st edition, 337 pages, illustrated. World Health Organization (Columbia University Press, New York 27, New York), 1959. $6.75.

Le Paludisme Dans la Zone Pilote Antipaludique de Bobo Dioulasso (Haute-Volta, A.O.F.), by R. Choumara, J. Hamon, et al. No. 1, 125 pages, illustrated. Paris, Office de la Recherche Scientifique et Technique Outer-Mer, 1959.

Live Poliovirus Vaccines, by various authors (A Symposium). 713 pages, illustrated. Washington, D. C., Pan American Sanitary Bureau, (Scientific Publication No. 44), 1959. $5.00.

Mosquitoes of Medical Importance, by Richard H. Foote and David R. Cook. 158 pages, illustrated. Washington, D. C., Agricultural Research Service, U. S. Department of Agriculture (Government Printing Office), 1959. $2.50.

Sampling Microbiological Aerosols, by Harold W. Wolf, M.S., et al., Technical Development Laboratories, C.D.C., U.S.P.H.S., and Herbert M. Decker, M.S., et al., U. S. Army Chem. Corps. (Public Health Service Publication No. 686) 53 + v pages, illustrated. Washington, D. C., U. S. Public Health Service, (Government Printing Office), 1959. $0.45.

BOOK REVIEWS

The Ecology of Human Disease, by JACQUES M. MAY, M.D. 1st volume, 327 pages, illustrated. New York, N. Y., M. D. Publications, 1958. $7.50.

This book represents an effort to relate the various fields of geography, social sciences, and medicine. To achieve this the author has formulated a concept of "medical ecology," in which disease is defined as a convergence in time and space, within the person of the patient, of varied environmental stimuli, resulting either in ecologic adaptation and survival or in total maladjustment and death. This volume is the first of three which will consider the more important transmissible, degenerative and behavioral diseases; this first one is concerned, as will be the second, with the transmissible diseases.

The first portion of the book presents definitions of terms and delimitations of areas of consideration necessary to the ecological descriptions of the specific diseases. The latter portion, approximately two-thirds of the volume, considers individually the ecology of a number of transmissible diseases (cholera, brucellosis, poliomyelitis, tuberculosis, leprosy, shigellosis, salmonelloses, amebiasis, yaws, certain two-factor nematode infections of man, scarlet fever, measles, and trachoma). Each of the chapters on specific diseases is well documented, with a total of over 800 references. A useful index is also included. The volume should be of considerable interest and benefit as a source of information in, and especially as a stimulus to further consideration of, this new concept of the ecology of human disease.

It is obviously impossible to consider fully and with complete accuracy of detail the geographical distribution and factors affecting the various diseases discussed in so brief a work. While this book cannot be considered as a complete description of the worldwide ecology of disease, it seems commendable as an introductory work—a beginning toward a global concept of disease. While the reviewer could not comment on the author's treatment of all diseases, in the discussion of some of those with which he is familiar a number of inaccuracies and gaps in information appear. There is occasional use of isolated instances, often rather insignificant ones, as examples of worldwide conditions. In giving prevalence figures the author

frequently provides the total numbers of cases reported without percentages or the size of the sample involved.

While some may not consider this work quite equal to the "blazing pennant announcing a new perspective in the study of disease ecology," as promised by Martí-Ibáñez in his expansive *Foreword*, it still can fulfill its stated purpose. It should be quite useful to geographers, anthropologists, and others not insistent on completeness of detail but interested in a general consideration of the geography of disease.

G. M. JEFFERY

Parasitology (Protozoology and Helminthology) in Relation to Clinical Medicine, by K. D. CHATTERJEE, M.D., 2nd Edition, 188 pages, illustrated. Published by the author, 6, Amrita Banerjee Road, Kalighat, Calcutta 26, India. 1959. Rs. 17.50

After publishing the large treatise entitled *Human Parasites and Parasitic Diseases*, the author prepared a reduced version containing the basic facts for medical students under the title *Parasitology (Protozoology and Helminthology) in Relation to Clinical Medicine*. The latter was rapidly exhausted, which made possible the present second edition. New information and new illustrations have been added to the second edition without materially increasing the size.

The book is divided into four sections. The introduction is concerned with terminology and general principles. The fourth section is an appendix which deals with techniques, culture methods, protozoa of uncertain status, and two full-page illustrations showing the location of parasites within the body. The two sections on protozoology and helminthology constitute the main part of the book. In these sections the parasites of man have been classified and described under the following headings: geographical distribution, habitat, morphology and life cycle, pathogenicity and clinical features, diagnosis, therapeusis and prophylaxis.

Outstanding features of the book are the many illustrations; 16 of these are in color, including plates showing the fixed-tissue stages of three species of human malaria. The large, well-labeled, life-cycle figures make easier to understand host-parasite relationships which often are quite complicated. Also valuable are the text tables listing the differential characteristics of certain parasites.

Many facts and descriptions appear to be greatly simplified which, in general, add to the appeal of the book; but errors are present, some of which may be due to oversimplification. In one table under a column entitled "Pathogenic Lesions," in some instances are shown only symptoms or descriptive names. The statement that *Enterobius vermicularis* eggs are not found in stool specimens should be modified to indicate that they are occasionally found. Among statements which need modification are: that limiting the distribution of *Plasmodium ovale* to East Africa and the Philippines; that stating the youngest observed age of the exoerythrocytic stages of human malaria to be 5 days; referring to *Trypanosoma rangeli* as a new species although it was described in 1920; saying that malaria paroxysms start either in the forenoon or early afternoon (actually they may start at any time); indicating a 48-hour periodicity for *P. ovale* malaria; and stating that immunity to malaria disappears with the elimination of the parasites. Some workers in the field of toxoplasmosis may not agree that the modes of transmission are as definite as indicated in the text.

Certain misinformation which has long existed is retained here also. These include the 48-hour life cycle for *P. vivax* in man and the indication in the text and in a figure that *P. falciparum* parasites disappear from the blood stream during the afebrile periods of the acute attack.

The amount of the information on the various aspects of the specific parasites in general appears to be in keeping with the length and purpose of the book. The subject of therapeusis is greatly abbreviated with the commonly used drugs shown without dosages. Some recently developed drugs have been included, such as dithiazanine, which is useful against *Trichuris*, but primaquine, known since 1950 to be useful for the radical cure of malaria, is not listed.

Overall, the material is clearly and attractively presented. Students will appreciate its clarity and straightforward approach. Parasitologists will find interesting the viewpoint and presentation of the subject by an authority with a rich background of experience in India. This book should serve well the purpose for which it was written and this edition should be as popular as its predecessor.

MARTIN D. YOUNG

A Textbook of Medicine, by RUSSELL L. CECIL, M.D., Sc.D. AND ROBERT F. LOEB, M.D., Sc.D., D.HON. CAUSA, LL.D., editors, with associate editors. 10th edition, 1665 pages, illustrated. Philadelphia and London, W. B. Saunders Co., 1959. $16.50—1 vol., or $20.52—2 vols.

The tenth edition of this now classic, "A Textbook of Medicine," is available in both one and two volumes. In the preface the editors list a great number of articles in the 10th edition which were not covered in previous editions. The contributors are outstanding investigators and clinicians in a

wide variety of medical subjects. This reviewer was particularly interested in the book from the standpoint of its suitability as a text for clinicians primarily interested in tropical medicine. In this connection it can be emphasized that the infectious diseases of both temperate and tropical zone importance are exceedingly well represented in the text. This is particularly true in the case of the mycoses. Infections caused by parasites are covered in many instances as thoroughly and in as great depth as may be found in some textbooks of tropical medicine. The deficiency diseases and diseases of metabolism are especially important for the practitioner of medicine in the tropics and these are very well covered. On the whole, one would have no hesitation in recommending this text as a practical reference for use by the student or practitioner of tropical medicine in conjunction with supplemental material dealing in greater length with the parasites themselves. The sections which this reviewer examined in detail were well written and free of typographical errors, although there is an amusing error in the section on malaria where the "cold" stage of an acute attack of malaria is referred to as the "cold storage."

HARRY MOST

Assistance Technique en Médecine Africaine, by Docteur RENÉ DEVIGNAT, Directeur de l'École de Médecine Tropicale et de l'École d'Assistance Médicale Indigène, à Elisabethville, Belgian Congo. 1st edition, 167 pages, illustrated. Paris, Masson et Cie, 1958. 1.600 fr.

Intended for students in schools of tropical medicine, for the Indigenous Medical Service and for auxiliary medical personnel in tropical Africa, this volume, covering Bacteriology, Virology, Mycology, Protozoology and Hematology, is the first of 3 similar manuals. Volumes 2 and 3 will deal with Helminthology and Entomology, and with Rodentology, Malacology and Herpetology, respectively.

The 3-page introductory chapter of Volume 1 mentions distinctive characters of bacteria, rickettsiae, viruses, mycobacteria, spirochetes, protozoa, yeasts and molds. Chapter 2 outlines diagnostic microscopy, with formulas for common stains, etc. Cultural methods are not detailed. Mention is made of diagnostic inoculation of animals and embryonated eggs. Chapter 3 deals with viruses in 7 pages; Chapter 4 with rickettsiae in 4 pages. Bacteria are sketchily outlined in Chapter 5; the mycobacteria in Chapter 6 (4 pages); the yeasts and molds and mycoses in Chapter 7 (6 pages). Chapters 8 (spirochetes) and 9 (protozoa) occupy about one third of the book. In the outline of syphilis the *T. pallidum* immobilization test is mentioned but not the more recent specific complement fixation test. Chapter 10 discusses clinical hematology.

Evidently the author expands on the most familiar topics or those considered most important. Thus, yellow fever is given 16 lines, dengue 6, the viral encephalitides 12. Tetanus is mentioned but not, what would seem more of a problem among tropical indigenes, tetanus neonatorum. In contrast, toxoplasmosis is given 1¼ pages, malaria 10 and trypanosomiasis 4.

These variations result from differences in mode of presentation. Most of the book suggests a lecturer's outline, leaving *much* to be interpolated by the lecturer, or by the hearer from his own background if he is a medical graduate.

The drawings, including color plates, are amateurish and suggest blackboard sketches which, perhaps are all that are required in a work of this sort. The book is in no sense a treatise, a textbook or a laboratory or diagnostic working manual.

Despite shortcomings in content and some factual errors (e.g., Dick and Schick tests are not based on allergy, although this is not very important here since scarlatina and diphtheria are virtually unknown in tropical Africa) the book can serve its purpose of making available to the lecturer in accelerated courses the essentials of the diverse matters generally classified as tropical medicine. It provides a "memory-aid" on microbiology for the physician and a guide especially adapted to the needs of itinerant missions and of rural medical groups in remote and less Europeanized parts of the Belgian Congo and elsewhere in tropical Africa.

M. FROBISHER

Blindness in West Africa, by F. C. RODGER, M.D., Ch.M., D.O.M.S., F.R.S.T.M. & H. 1st. edition, 262 pages, illustrated. London, H. K. Lewis & Co., Ltd. (for the Royal Commonwealth Society for the Blind), 1959. £ 3-10-0.

This monograph is the most comprehensive and critical study of the prevalence and etiology of blindness in any tropical region. Comprehending three extensive areas in West Africa, viz., Northern Ghana, North Nigeria and adjacent North Cameroons (British Trust Territory), the investigation by the author covered 4 years of intensive study in a random sampling of many villages, most of which were in back country difficult of access.

In Part I, following a description of the areas surveyed, their geographical location, topography, climate, flora and fauna, the racial and tribal characteristics, customs and pursuits of the inhabitants, Rodger reports on previous surveys,

then proceeds to present the plan of his research and his findings. Among the common eye diseases observed were trachoma, purulent conjunctivitis, punctate keratitis due to herpes febrilis and adenovirus following malaria, corneal ulceration as a sequela to smallpox and measles, leprosy, syphilis, onchocerciasis, traumatic damage, senile cataract and avitaminosis A. The chief causes of blindness are trachoma, senile cataract, smallpox and measles and, in hyperendemic foci, onchocerciasis.

Conservative estimate of the incidence of blindness for these West African countries is 1 per cent (Northern Ghana, 3.0%; North Nigeria, 0.8%; North Cameroons, 1.8%), while in some hyperendemic foci of onchocerciasis in Northern Ghana it reaches the almost incredible figure of 18 per cent, compared with 0.2 per cent for Europe.

The total blind population of these three countries is estimated to be 200,000 (30,000 in N. Ghana, 150,000 in N. Nigeria, and 5,000 in N. Cameroons). Individuals under 18 years of age constitute 12 per cent of the total blind in N. Ghana, 10 per cent in N. Nigeria and 16 per cent in N. Cameroons. Analyzing the causes of blindness, the reader discovers that in N. Ghana the three most common are onchocerciasis (45.4%), trachoma (20.0%) and senile cataract (7.2%); in N. Nigeria, trachoma (30.6%), cataract (18.1%) and onchocerciasis (11.0%), and in N. Cameroons, trachoma (26.9%), onchocerciasis (23.6%) and cataract (17.6%). In many blind persons the etiologies are multiple. In much of the northern territories, in contrast to the more southern tropical rainforest areas, there are drought and near famine for 4 months preceding the annual rains. The majority of the population investigated have an adequate caloric intake for the other 8 months but deficiency in certain essential amino acids, vitamin A, riboflavin and vitamin C; of these the greatest lack is vitamin A. Blindness in approximately 10,000 persons in the regions surveyed results directly or indirectly from malnutrition.

Part II of the monograph is devoted to "Onchocerciasis and Other Filarial Diseases." For 2 years after Robles (1917) attributed blindness in Guatemala to onchocerciasis there was no reported association between *Onchocerca volvulus* and blindness in tropical Africa. After Hisette (1938) demonstrated ocular involvement in this infection in the Belgian Congo a reappraisal of onchocerciasis in tropical Africa gradually occurred. Rodger found this infection to be one of the most frequent and, in hyperendemic areas, the most important cause of blindness, invading and producing pathology in all tissues of the eye. To obtain evidence of circulating microfilariae of *O. volvulus* in the individual, and density of the infection in the community, the author took right and left biopsy snips from the calf, thigh, chest, neck and conjunctiva. With few exceptions the largest numbers of microfilariae in adults were found in the lower extremities and successively smaller numbers ascending to the eye. In only seven of 1,100 cases of all ages were conjunctival samples positive when skin biopsies were negative. By comparing these data from different geographical areas it was possible to conclude that in the most heavily endemic village one half the population became infected by the 7th year of age, while in a mildly endemic area this proportion was not reached until age 48.

Palpable parent *Onchocerca* nodules on the head were found to be much more common in persons under 6 years of age than in older individuals. There was no significant association between the mean number of palpable nodules (0.5 to 4.0 in 14 different areas) on the one hand and the density quotient or infectivity rate of microfilariae on the other.

In N. Ghana, between 11° and 9° N., the calculated microfilarial prevalence rate of onchocerciasis was 60 per cent; in N. Nigeria, between 12° and 8° N., 10 per cent, and in N. Cameroons, between 9° and 7° N., 30 per cent. Infection is limited to areas below 4,000 feet, above which the vector *Simulium damnosum* does not breed. Frequently, persons without palpable nodules had positive skin biopsies. Paradoxically, this condition most often occurred in communities with a low density quotient of circulating microfilariae. Rodger explains this finding on the basis of undiscovered unencapsulated parent worms, and, in turn, attributes lack of encapsulation to deficiency of vitamin A. He concludes that the adult worms may live for 10 to 15 years, and the microfilariae 3 to 9 months. He states that "the number of microfilariae in the skin in a heavily infected case may run into billions."

The author approaches his consideration of eye pathology by reference to the topographic prevalence of the nodules. In children in hyperendemic villages of N. Ghana 58 per cent of the nodules were located on the head, with a short route for the microfilariae to the eye and hence much greater risk of blindness. In adults the path is from the lower extremities or trunk upwards, so that few microfilariae invade the eye except in very heavy infections. In Eastern Nigeria with a high infectivity rate and a high average number of nodules, there was no blindness, since the microfilariae were confined below the shoulders. This population has a high vitamin A content in its diet. Rodger associates nodule formation with vitamin A sufficiency, and, conversely, failure to produce fibrous encapsulation around the parent worms with avitamin-

osis A. On the basis of careful study of sections of 20 enucleated eyes damaged by *Onchocerca* (the largest collection ever assembled) and experimental tests as well as clinical observations, he is convinced that the inflammatory lesions in the eye and their sequelae result from death of the microfilariae which have invaded the tissues of this organ, with an underlying deficiency in vitamin A.

Diagnosis of blood films taken during the survey indicates that *Acanthocheilonema perstans* is more common than *O. volvulus* in N. Nigeria and N. Cameroons, less prevalent in N. Ghana; that *Wuchereria bancrofti* has appreciable incidence in N. Ghana and N. Nigeria, is uncommon in N. Cameroons, and that *Loa loa* occurs in N. Nigeria and N. Cameroons but not in N. Ghana. *A. streptocerca* was not encountered in the study areas.

The volume is provided with a magnificent color frontispiece, 96 illustrations in the text, several of which are excellent photomicrographs of sections of different tissues of the eye containing microfilariae, tables presenting detailed findings, several maps and nine appendices. The format is pleasing, the glossy paper is well chosen for reproduction of the numerous photographs, and there are relatively few typographical errors. Although there is necessarily some technical terminology which is best appreciated by the ophthalmologist, the author is entirely at home with respect to the various stages of *Onchocerca volvulus* and the epidemiology of the disease produced by this filaria. Critics may question some of his views but his basic observations and conclusions are sound.

ERNEST CARROLL FAUST

Report of the First Institute on Clinical Teaching: 6th AAMC Teaching Institute, HELEN H. GEE AND JULIUS B. RICHMOND, Planning Committee, and E. S. NOURSE, editors. 233 pages, illustrated. Evanston, Ill., Association of American Medical Colleges, 1959. Cloth $3.00, paper $2.00.

Since 1953, the Association of American Medical Colleges has conducted an annual Teaching Institute, attended by appropriate representatives of each of the medical schools of the U.S.A. and Canada, plus a list of distinguished participants. Such institutes have covered the problems of teaching Physiology, Biochemistry, and Pharmacology (1953); Pathology, Microbiology, Immunology, and Genetics (1954); Anatomy and Anthropology (1955). In 1956 and 1957 the Institute discussed the ecology of the medical student, selection, and evaluation procedures, etc.

The 1958 Institute is the first of a series on Clinical Teaching. The report of the 1958 Institute consists of a number of papers on the objectives of clinical teaching, on the setting of clinical teaching, about the student, patient, and teacher, and a discussion of extramural forces influencing medical education.

Objectives set by Edgar Hull, Henry Bakst, and Carl Moyer ranged from pragmatic and encyclopedic emphases to the need for attention to social forces, and returned to profound but turbulent mid-stream ideas, with reliance upon the scientific method. Rescue from chaos was attempted by Dana Atchley who prescribed "Science, Art, and Heart" in a compounded educational prescription.

Stewart Wolf introduced ideas from the numerous participants, most of whom concerned themselves with education versus training. The behavioral scientists were welcomed to the medical faculty; first to learn and later to teach.

Part II explored the setting of clinical medical education. The teaching hospital was explored, praised, damned, audited (in regard to cost of operation), and discussed from one extreme, as a trap for use of students as servants rather than as scholars, to the other extreme as a Mt. Olympus of accumulated medical knowledge in danger of obliteration by the storm clouds of prepaid voluntary health insurance.

Part III covered the multiheaded dragon of the learning process as it involved the student-participant, the teacher-scholar, and their mutual focus of interest, the patient. Strong sentiment developed to delete "teaching" in favor of "learning" in the vocabulary. The teacher-scholar was motivated to set a proper demonstration of an elaborate yet practical example of the accomplished "learner," and the behavioral scientist on the panel garnished the discussion with witty yet noncontributory illustrations.

The system of Swedish medical education was contrasted with our own by the Chancellor and Professor of Surgery of the University of Lund in a most charming manner.

Part IV explored extramural forces affecting medical education. Charles Frankel (philosopher) encouraged the development of special skills in discrimination between the relevant and the irrelevant in medical education, pointed out the need for practical judgments built on comprehensive yet precise generalities, and came out strongly for specialization as the only available means of making systemic improvements in our educational process.

Seymour Harris (economist) reviewed the big-business aspects of medicine. He pointed out that 1 per cent of a lifetime income of the physician would easily finance his medical education, and listed wastes in the use of drugs and other health appliances, and in hospitalization due to easy insurance plans. He recommended more student scholarships and higher pay for outstanding faculty members.

Ralph Tyler (behavioral scientist) developed an apology and a justification for his specialty, recommending greater use of his colleagues in medical education, an opinion not viewed with enthusiasm by most seasoned medical educators.

This volume ought to be given a place on the reading shelf of all medical faculty members along with the other Institute Reports. The papers printed are provocative and challenging, the discussions (which are all too sparse in this text) are uniquely valuable. The appendix is well furnished with data about clinical medical education in the U.S.A. and Canada, and alone is worth the purchase price of the volume. A second report on Clinical Teaching is forthcoming from the 1959 Institute.

J. R. SCHOFIELD

Water Supply for Rural Areas and Small Communities, by EDMUND G. WAGNER, Rio de Janeiro, AND J. N. LANOIX, Geneva. 1st edition, 337 pages, illustrated. Geneva, World Health Organization (Columbia University Press, New York 27, New York), 1959. $6.75.

Since 1951, the World Health Organization has published a series of monographs on important aspects of human health. The authors of the 43 monographs that have appeared to date have been staff members assigned to this duty or consultants engaged specifically for this purpose. In Edmund G. Wagner and Joseph N. Lanoix, the Division of Environmental Sanitation has found a team that brings to its task the experience gained in many lands, first under the aegis of their own governments and later under that of international health authorities including, in the case of Mr. Lanoix, WHO itself.

Water Supply for Rural Areas and Small Communities is a companion volume to *Excreta Disposal for Rural Areas and Small Communities*, by the same authors. Both books, however, reflect not only the team's competency but also, in a sense, the experience of engineers throughout the world, because the manuscripts have been reviewed by regional public health engineers, physicians, and sanitation personnel stationed in widely scattered places: to the number of 38 in this instance, for example.

The aim of this monograph is to assist government officials or, more specifically, health administrations in the prevention of illness and death from water-borne diseases in the environments to which the text is directed. This aim the monograph should fulfill admirably by its three major divisions: Development of water-supply programs (44 pp.), Installation of various types of water supply systems (170 pp.), and Management of water-supply systems (24 pp.). The principles of development and management are well stated, and the technical discussion of both ground and surface water supplies is sufficiently detailed to be of immediate help to builders, especially if they will avail themselves of the tables and instructions presented in the 74 pages of annexed material. Annexes 5 and 7 prepared with the assistance of W. E. Wood, Chief Water Engineer, Ministry of Works, Nigeria, should receive particularly wide commendation.

The health aspects of water supply and means for the preservation of water safety and attractiveness are stressed throughout the monograph; the hydraulics of small systems are covered quite adequately; and the limitations of water treatment under rural conditions are explained realistically. Least effective, perhaps, is the consideration of the hydrology of water supplies which is the ultimate determinant of their quantitative adequacy. The reader will miss in particular a presentation of ways and means for ascertaining the yield of both surface-water and ground-water sources. In the reviewer's opinion, such a discussion lies well within the scope of a treatise of this kind and compass. The user of the hydraulic tables in Annex 3 should be warned that the capacity values in the second column of all tables for 1½-inch and larger pipe are "million gallons per 24 hours" not "gallons per 24 hours" as printed. Otherwise typographical errors or omissions are relatively rare. The workmanship of printing and binding is excellent.

For the overall value of this monograph to human progress a quotation from a committee report of the American Water Works Association, given on page 233, appears to be particularly apt: "It is extremely important to remember that small water systems do not imply either small problems or small responsibilities."

GORDON M. FAIR

Le Paludisme dans la Zone Pilote Antipaludique de Bobo Dioulasso (Haute-Volta, A.O.F.), by R. CHOUMARA, J. HAMON, ET AL. No. 1, 125 pages, illustrated. Paper covers. Paris, Office de la Recherche Scientifique et Technique Outer-Mer, 1959.

An experiment in malaria eradication by residual spraying is reported in this, the first of a series of reports on various projects by ORSTOM, the French governmental agency for overseas scientific and technical research. Forty-one tables, 2 maps, and 10 graphs, as well as French, English, Spanish, and German summaries, are included, with an introduction by the Director General of ORSTOM and a preface by M. Vaucel, Inspector General of Overseas Pasteur Institutes.

The experiment was at Bobo Dioulasso, where malaria, chiefly falciparum, was highly endemic. The population of 50,000 in 100 villages was agricultural and families usually divided their time between village dwellings and huts out in the fields. The chief vectors were *A. gambiae*, *A. funestus*, and *A. nili*, with help from *A. coustani*. Spraying, begun in 1955, was mostly with DDT but in two villages was with dieldrin. Some DDT and BHC spraying had been done in 1953–1954.

Parasite indexes in unsprayed villages were 76.9 in 1956 and 50.6 in 1957; in DDT-treated, 75.5 in 1953 and 50.6 in 1957; in dieldrin-treated, 74.0 in 1955, and 63.8 in 1957. Infant parasite indexes in DDT-treated villages were 39.0 Nov. 1955, 37.6 Feb. 1956, 18.5 Feb. 1957, and 14.7 Feb. 1958.

Obviously, malaria transmission was not interrupted. Vaucel pointed to a repelling effect of DDT on *A. gambiae*, out-door feeding habits of *A. nili* and *A. coustani*, coincidence between hours of feeding by vectors and hours when the people were outside, role of secondary vectors, and resistance of *A. gambiae* to dieldrin, as some of the reasons for lack of success. To what extent such factors as incomplete coverage, insufficient organization, inactivation of insecticide deposits, and chemotherapy were involved is not clear. Experience elsewhere in Africa has demonstrated the need for larger areas, longer periods, and tighter organization and inspection in malaria-eradication experiments.

P. F. Russell

NOTICE TO CONTRIBUTORS

The Journal welcomes papers which contribute to the purpose of the American Society of Tropical Medicine and Hygiene. This is the advancement of knowledge of tropical and subtropical medicine and hygiene through investigation, prevention, treatment and control of diseases and deficiencies. It draws upon medicine, biology, public health, nursing, engineering and allied fields of research and application.

The American Journal of Tropical Medicine and Hygiene combines the American Journal of Tropical Medicine and the Journal of the National Malaria Society and therefore has a special concern with the investigation, therapy and control of malaria in all parts of the world, as well as a broad interest in general parasitology and arthropod-borne diseases.

Papers are ordinarily published in the order of reception, which will entail a certain delay in their appearance because of the contractual limitation on the number of journal pages available in any year. Accepted articles which raise no question of scientific priority but for which immediate publication seems important will be inserted in the next available issue of the journal if the author or sponsoring institution will pay the cost, estimate of which may be obtained from the editor. The insertion of such articles does not affect publication of other manuscripts awaiting their turn, since the inserted articles constitute additional pages in the volume without additional cost to the subscriber.

Publication will be facilitated if the following suggestions are followed:

1. *Manuscripts* should be typewritten in *English* using *double spacing* on *one side* only of 8½ x 11 inch paper and submitted *in duplicate*. Double-spacing should be used throughout the manuscript including the list of references, footnotes, and quotations.

2. The author should supply, on a separate sheet, a short title not over 50 letters for use as a running head. Also on separate sheets type (a) the authors' names and affiliations; (b) tabular matter; (c) case histories; (d) quotations; (e) formulas and other subsidiary matter in the text; (f) footnotes; (g) references; (h) legends for illustrations; (i) displayed formulas occupying more than 1 line of type, even if using the same size type as the main text.

3. Legends should be separate and not attached to, or written on, the illustration copy.

4. Positions for tables and figures in the text may be indicated on the margin of the text pages.

5. All papers should end with a brief summary.

6. *Tables* should be typed on separate sheets and be serially numbered in Arabic numerals.

7. *Figures* and *photographs* should be protected with cardboard to avoid creasing in the mails. Only glossy prints can be used. Line drawings and graphs should be made with India ink and submitted on white (not cross-section) paper. Colored illustrations can be reproduced only at the expense of the author.

8. References should be cited by consecutive numbers in the text, and so numbered and listed in the list of references at the end of the article, without regard to alphabetical arrangement. References should be written as follows:

> 1. Reed, L. J., and Muench, H., 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, **27**: 493–497.

9. *Proprietary names* of drugs or chemicals registered by commercial pharmaceutical firms may not be used in the text if there is a generic name available in the U. S. Pharmacopoeia, New and Nonofficial Remedies of the A. M. A., or the latest editions of standard medical dictionaries. In certain cases where the generic name is relatively unknown, the registered name may follow it in parentheses in the title, at the first citation in the text, and in the summary.

10. **N. B. The address to which proofs are to be sent** should be indicated at the top of the first page of the manuscript. Delay will result if proofs must be forwarded.

Estimating costs of reprints. Where the cost of reprints must be known in advance of publication in order to secure prior approval for their purchase, the Editor will be glad to furnish an estimate of the number of pages which the article will occupy in the Journal.

DIFCO LABORATORY PRODUCTS

BIOLOGICS CULTURE MEDIA REAGENTS

Media for Standard Methods

Culture Media *Dehydrated and Prepared*

Microbiological Assay Media

Tissue Culture and Virus Media

Bacterial Antisera and Antigens

Diagnostic and Serological Reagents

Sensitivity Disks Unidisks

Peptones Hydrolysates Amino Acids

Enzymes Enrichments Dyes Indicators

Carbohydrates Biochemicals

DIFCO

over 60 years' experience
in the preparation of Difco products assures
UNIFORMITY STABILITY ECONOMY

•

Complete Stocks *Fast Service*

•

Descriptive literature available on request

DIFCO LABORATORIES **DETROIT 1 MICHIGAN USA**